Review

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Evidenc against th hyp thesis that antibodies to the inner core of lipo-polysaccharides in antisera raised by immunization with enterobacterial deep-rough mutants confer broad-spectrum protection during Gram-negative bacterial sepsis

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Summary Antisera to rough enterobacterial mutants of chemotypes Ra, Rc, and Re have been reported to confer broad-spectrum protection against wild-type smooth strains. It has been hypothesized that binding and neutralization of lipopolysaccharides (LPS) by antibodies to common core epitopes underlies such protection. This review summarizes experiments by our laboratory and others that do not confirm this concept and proposes reasons for the divergent results. Studies indicating broad-spectrum protection by rough-mutant antisera often had defects in experimental design or methodology. These include the failure: (i) to use matched pre- and postimmune sera from the same donors to control for variable protective activity of normal sera; (ii) to exclude the role of natural and polyclonally stimulated antibodies with proven protective activity against the infecting bacterial strain (e.g. O-specific, capsular, *Pseudomonas* exotoxin A); (iii) to exclude protective effects of acute-phase serum factors; (iv) to exclude protective effects of endotoxin contamination after adsorption or fractionation of antibody preparations; (v) to use non-boiled bacteria and LPS not subjected to acid-hydrolysis or gel-fractionation, and to exclude nonspecific adsorption, to demonstrate physiologically meaningful binding of rough-mutant antibodies to smooth enterobacteria and their LPS.

INTRODUCTION

Over the past 3 decades, there has been a revival of interest in the potential of rough enterobacterial mutant vaccines to enhance survival from Gram-negative bacterial sepsis. This interest is based on the fact that the core polysaccharide and glycolipid regions of LPS are much more conserved across the various enterobacteria than the O-polysaccharide region so that a single polyclonal antiserum or monoclonal antibody might confer broad-spectrum protection against Gram-negative bacterial sepsis. Most investigators have employed the J5 mutant of *Escherichia coli* O111:B4 (an Rc chemotype) or the R595 mutant of *Salmonella minnesota* (an Re chemotype) for generating protective antisera. Since the 1970s, our laboratory has been concerned with the lack of convincing evidence that J5 and R595 polyclonal rough-mutant antisera are cross-protective because of antibodies to common core LPS epitopes. This review will present a critical evaluation of this evidence. It will detail problems in

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methodology that we and others have encountered that can make it appear that antibodies to LPS common core epitopes in J5 and R595 rough-mutant antisera are the protective factors. Those studies using monoclonal antibodies (MAbs) to explore broad-spectrum protection will not be addressed except as they directly pertain to the polyclonal data.

R ugh-mutant vaccines: historical perspective

The possibility that antigens in rough-mutant enterobacteria might provide the basis for an effective vaccine against lethality from sepsis caused by wild-type smooth strains of Gram-negative bacteria was initially explored in the 1920s.[1,2] Classic studies were reported by Arkwright in 1927.[2] Guinea pigs were immunized with phenol-killed motile and non-motile rough-mutant strains of *Bacillus typhosus* and *B. paratyphosus*, as well as with the parental smooth wild-type strains, and 1–3 weeks later challenged i.p. with the homologous viable smooth parental strain. Arkwright concluded: 'used as killed vaccines the [bacterial] forms containing the smooth heat-stable antigen produce a high state of immunity...R [rough] heat-stable antigen appears to have little or no value'.[2] He also discussed the studies of typhoid vaccine efficacy by Weber[3], stating, 'my experiments confirm his [Weber's] view...that spontaneously agglutinating (i.e. R) cultures should not be used'.[2] In 1928, Ibraham and Schutze explored the ability of a heat-killed rough-mutant *Salmonella typhimurium* vaccine to protect mice against i.p. challenge with the viable smooth parental strain. They concluded: 'only vaccine containing agglutinogenically active H and O antigen in combination has been shown to be effective... Vaccines containing only R or H + R antigens may be regarded as ineffective'.[4]

In 1941, De Torregrosa and Francis observed that mice could be protected against intracerebral challenge with a smooth strain of *Haemophilus influenzae* type b if the organisms were preincubated for 30 min with homologous antiserum diluted 1:500, whereas antiserum against a rough strain of *H. influenzae*, even minimally diluted (1:1), conferred no protection.[5] In 1968, Kenny and Herzberg reported that a live rough-mutant *Salmonella enteriditis* vaccine provided mice with sustained protection against i.p. challenge with the parental smooth strain; such protection did not extend to challenge with *S. typhimurium* and correlated with increments in bactericidal antibody titers against *S. enteriditis*.[6] That same year, Holme et al. vaccinated mice with heat-killed smooth *S. typhimurium* and its rough-mutant Ra, Rb, Rc, and Rd chemotypes. They concluded that, in contrast to vaccines prepared from the smooth strain, 'vaccines prepared from the [rough] mutant strains did not confer protection in mice against [i.p.] challenge with viable [parental] *S. typhimurium* 395MS'.[7]

Most of the early studies involved active immunization of mice or guinea pigs with rough-mutant *Salmonella* vaccines, followed by i.p. challenge with the wild-type strains. It is now known that cellular immune mechanisms are important in the resistance of mice to *Salmonella* infections.[8] A number of investigators have shown by passive transfer, however, that O-specific antibodies alone are highly protective when Salmonellae are given by the i.p. route.[8–10] Thus, the failure of vaccination with rough-mutant *Salmonella* strains to protect mice and guinea pigs against i.p. challenge with wild-type strains indicates not only that cellular immune mechanisms against the parental strain were not effectively activated, but also that, in contrast to O-specific antibodies, the antibodies raised to R-form LPS of the parental strain were incapable of protection.

Renewed interest in the potential of rough-mutant vaccines followed the work of Chedid and co-investigators who reported in 1968 that an equine antiserum raised against an Ra chemotype of a rough mutant of *S. typhimurium* was capable of protecting mice against lethality from the Caroli strain of *Klebsiella pneumoniae*.[11] This work stimulated numerous subsequent studies of rough-mutant antisera as an immunotherapeutic approach to the problem of Gram-negative bacterial sepsis; some are still in progress. Antisera to an Ra chemotype of *S. typhimurium* (TV119), to Rc chemotypes of *E. coli* O111: B4 (J5) and *S. minnesota* (SF1119), and to an Re chemotype of *S. minnesota* (R595) have all been reported to be effective.[11–21] Antisera to these rough mutants were also reported capable of providing broad-spectrum passive protection against the lethal activity of LPS isolated from heterologous wild-type smooth enterobacteria (S-form LPS); indeed, such broad-spectrum protection against heterologous S-form LPS was claimed to be virtually as effective as that provided by O-specific antiserum to its homologous S-form LPS.[22–24] Of especial importance was the proposal that such broad-spectrum protection against LPS constitutes the underlying mechanism for the broad-spectrum protection conferred by rough-mutant antisera against mortality due to Gram-negative bacterial infections.[25,26]

Re-evaluation of the evidence for broad-spectrum protection by antisera to rough mutants

There are a number of puzzling observations concerning the above-cited reports of broad-spectrum protection by polyclonal antisera to the Ra, Rc, and Re chemotypes of rough-mutant enterobacteria.[11–26]

1. No significant protection was observed by the earlier investigators[1–7], even though active immunization was

performed with vaccine strains of rough-mutant enterobacteria whose chemotypes presumably would have been encompassed within the Ra to Re range. In one of these studies, the vaccine strains were proven to encompass the entire Ra to Rd range.[7] Furthermore, even protection against the homologous parental strain was not seen by the earlier investigators unless titers of antibody specific to the parental serotype had increased.

2. Broad-spectrum protection was reported to be conferred by antiserum to an Ra chemotype of *S. typhimurium*,[11] yet other investigators reported that only Re and Rd2 chemotypes of rough *Salmonella* mutants were capable of inducing cross-protective antisera.[12]

3. Passive immunization of mice with rabbit antisera to the R595 (Re) rough *S. minnesota* mutant was reported to confer significantly greater protection against challenge with viable *E. coli* than did rabbit antisera to the parental smooth *S. minnesota*; however, no significant differences in protection were seen when mice were actively immunized with these *Salmonella* strains.[12]

4. After mice were actively immunized with the R595 *Salmonella* mutant, no broad-spectrum protection was found until titers of circulating hemagglutinating (HA) antibody to the Re LPS were ≥ 1:160, whereas protection by passive immunization with rabbit antiserum occurred when titers of circulating antibody were only 1:20.[12] (We recognize the possibility that this discrepancy might simply reflect differences between protective activities of murine and rabbit antibodies.)

5. Broad-spectrum protection in granulocytopenic rabbits actively immunized with the R595 mutant was attributed to antibodies to the Re LPS even though comparable titers of polyclonal serotype-specific antibodies, which possess considerably greater protective activity,[12,15,16,27] were evoked by the immunization.[17]

6. The protection reported against meningococcal LPS by J5 rabbit antisera was found to reside entirely in the IgG fraction,[28] whereas protection by R595 rabbit antiserum against *S. typhi* LPS was reported to reside almost entirely in the IgM fraction even though the immunization schedules were virtually identical.[29]

A particularly puzzling observation was that by Braude and Ziegler, who noted that 'equal protection occurs against heterologous LPS with antisera generated against smooth and rough LPS, providing further proof that O antibody is not necessary for preventing death from endotoxin'.[30] Since antisera generated against S-form LPS are unreactive with the LPS Rc core of J5 *E. coli*, with the Rd or Re core of *Salmonella*, or with lipid A,[31–34] this observation strongly implies that factors in J5 and R595 antisera other than antibodies to J5 or R595 LPS core epitopes or lipid A mediate their reputed broad-spectrum protection. Furthermore, if this observation were valid, it would raise a fundamental question concerning the basis of the preference for antisera to J5 and R595 rough mutants over antisera to smooth enterobacteria for broad-spectrum immunoprophylaxis of Gram-negative bacterial sepsis.

Because of these discordant observations, we re-evaluated the effectiveness of broad-spectrum protection by antisera to inner-core LPS epitopes of enterobacteria. Using murine models, we found that normal pre-immune sera obtained from heterologous species (rabbit and horse) provided significant but variable levels of broad-spectrum protection against wild-type viable enterobacteria. It is, therefore, crucial that any protection observed with immune sera be compared with that provided by pre-immune sera from the matched respective donors. Utilizing such essential controls, we could not demonstrate broad-spectrum protection against mortality due to wild-type enterobacterial infections (induced i.v. or i.p.) with rabbit antisera containing high titers of HA antibodies to Rc (J5) or Re (R595) rough mutant LPS.[35,36]

Studies by Ng et al.,[37] Peter et al.,[38] van Dijk et al.,[39] Trautmann and Hahn,[40] Vuopio-Varkila and co-workers,[41,42] and Baumgartner et al.[43] have all confirmed these negative findings. Welch et al. observed no protection against viable *E. coli* with rabbit antisera containing very high titers of antibody (1:20,480, assessed by passive hemolysis) to Re LPS core glycolipid.[44]

In the above studies, several points of particular interest should be stressed.

1. Peter et al. not only employed high-titered J5 rabbit antisera (HA titer to J5 LPS 1:10,240) to test its cross-protective activity in mice against a wild-type strain of *E. coli* (O6:K2:H1), but the testing was exhaustive: 25 separate studies were conducted using 343 control mice given saline or no pretreatment, 343 mice pretreated with the anti-J5 serum, and 345 additional control mice pretreated with matched pre-immune serum. Overall survival was 13%, 47%, and 44%, respectively, demonstrating not only the lack of cross-protective activity of the J5 antiserum but also the significant protective activity of pre-immune serum ($P < 0.001$) even though it possessed insignificant antibody titers (by HA and passive hemolysis) against J5 LPS or the wild-type challenge strain.[38] Without

the matched pre-immune serum controls, it might have been concluded that J5 antiserum was cross-protective.

2. van Dijk et al. reported 100% survival of mice challenged with E coli O111:B4 when pretreated with *E. coli* O111:B4 rabbit antiserum, and 0% survival when pretreated with J5 antiserum, i.e. the J5 antiserum provided no protection against the parental wild-type strain even though its ELISA antibody titer to the J5 mutant was high (1:12,000). The J5 antiserum also failed to protect against another non-encapsulated *E. coli* strain (F11).[39]

3. Baumgartner et al observed that J5 rabbit antiserum was not only incapable of protecting mice against lethality from *Pseudomonas aeruginosa* serotype 3, but was also ineffective against challenge with the parental *E. coli* O111 strain. Moreover, the J5 antiserum failed to inhibit the appearance of tumor necrosis factor (TNF) following administration of *E. coli* O111 LPS, whereas TNF was not detectable in animals pretreated with O-specific antiserum.[43]

It is of interest that Hodgin and Drews observed that normal rabbit serum significantly protected mice against sepsis due to *E. coli* O4 but, nevertheless, concluded that R595 rabbit antiserum provided better protection without specifying whether matched pre-immune serum controls were used.[16] Even without this essential control, their reported survival of mice given R595 antiserum and challenged with *E. coli* O4 was not significantly greater than that for mice given normal serum. Thus, their findings remain consistent with the above-cited negative findings. We suggest that the inability of antibodies to R595 LPS to confer broad-spectrum protection is actually strongly supported by an additional observation of Hodgin and Drews; i.e. mice actively immunized with the R595 mutant in incomplete Freund's adjuvant exhibited no more resistance to challenge with *E. coli* O4 than did mice given only the adjuvant, and in both cases resistance declined sharply by 6 weeks despite residual high titers of antibodies to the Re core glycolipid in the R595-immunized animals.[16]

J5 antisera from other animal species have also been found to be no more protective than normal serum. Wickstrom et al. demonstrated that although bovine J5 antiserum was more protective than saline against *E. coli* sepsis in calves, 'normal serum was almost as helpful as immune [J5] serum'.[45] Morris and Whitlock, in a double-blind prospective study, reported that pooled equine antisera to a combined J5 and R595 vaccine provided neonatal foals no more protection against naturally acquired Gram-negative septicemia than did pooled normal serum.[46] Selim et al. administered J5 immune bovine plasma selected for high J5 IgG ELISA titers to bovine neonates in a prospective double-blind clinical field trial; controls received non-immune bovine plasma or no treatment. The animals were evaluated during the next 3 weeks for naturally-acquired septicemia and colitis. It was concluded that the J5 plasma 'was not superior to control plasma or to no intervention in terms of calf morbidity and mortality'.[47]

Studies employing active immunization with the J5 *E. coli* mutant also failed to confirm effective broad-spectrum protection. Cryz et al. reported that although LPS from the J5 mutant was immunogenic in mice, active immunization failed to provide significant protection against any of five smooth strains of *P. aeruginosa* of different serotypes.[48] Sadoff et al. similarly observed that mice vaccinated with J5 LPS could not be protected against *P. aeruginosa* sepsis.[49] Sakulramrung and Domingue reported that active immunization of mice with J5 vaccine failed to protect against challenge with *K. pneumoniae* or *E. coli* O4.[50] Pennington and co-workers reported that guinea pigs actively immunized with the J5 rough mutant could not be significantly protected against intratracheal challenge with viable *P. aeruginosa*, *E. coli*, or *K. pneumoniae*.[51,52]

In the above studies,[16,35–52] the inability to demonstrate significant protection after either active immunization with rough-mutant bacterial vaccines or passive immunization with the resultant antisera could not be attributed to insensitivity of the assay system. With one exception, those studies that also evaluated the efficacy of serotype-specific antibodies demonstrated significant protection.[16,36,39–43,48–52] The exception involved an encapsulated organism, *E. coli* O7:K1:NM, whose lethality in mice was reduced only by capsular antibody.[44]

Since no significant broad-spectrum protection against enterobacterial or *Pseudomonas* sepsis could be demonstrated either by active immunization with the J5 or R595 rough mutants or by passive transfer of antisera to these mutants by our laboratory or those indicated above,[16,35–52] and since anti-LPS activity has been postulated as the basis of the broad-spectrum protection observed with such antisera by other investigators,[25,26] we re-evaluated the putative anti-LPS effect of these rough-mutant antisera. In these studies, all rough-mutant antisera were screened to preclude polyclonal increments in O-specific antibodies to the challenge LPS, and pre-immune sera from the respective donors were employed as controls. We found that rabbit antisera to J5 and R595 mutants, that possessed titers of antibody to the respective LPS core determinants comparable to or higher than those used by investigators who reported broad-spectrum protection against LPS,[24] failed to protect mice against lethality produced by LPS from heterologous smooth enterobacteria or even from the homologous

smooth parental strain. Moreover, the same negative results were obtained in actinomycin D-sensitized mice, in which protection against quantities of LPS as small as 0.25 μg could be assessed. In contrast to J5 and R595 antisera, O-specific antisera were consistently protective against the homologous S-form LPS. These results, all obtained with the use of the highest-titered antisera and tests within the sensitive dose-response ranges (1–2 LD_{50}) failed to support the hypothesis that antisera to J5 and R595 are capable of effective broad-spectrum neutralization of the lethal activity of S-form LPS.[27] More recent studies by Baumgartner et al. also failed to support this hypothesis, since J5 rabbit antiserum, in contrast to O-specific antiserum, proved incapable of protecting against either lethality in galactosamine-sensitized mice or the cutaneous Shwartzman reaction in rabbits elicited by the parental S-form LPS.[43]

A number of earlier studies are consistent with the inability of J5 and R595 rough-mutant antisera to offer effective protection against S-form enterobacterial LPS as a consequence of antibodies to inner core LPS epitopes. We observed that pretreatment of rabbits with large quantities of J5 rabbit antiserum (10 ml/kg) conferred statistically significant but physiologically minimal protection against fever induced by trace amounts (10 ng/kg) of a heterologous S-form LPS (*S. typhimurium*). The mean 3 h fever increment was 2.4°C in rabbits not pretreated with serum, 2.3°C in those pretreated with control *E. coli* O111 antiserum, and 2.0°C in those pretreated with J5 antiserum. Since additional controls receiving pre-immune sera from the J5 antiserum donors were not included, it is not possible to be certain that this small difference was based upon the activity of J5 antibodies. By contrast, as little as 0.5 ml/kg of O-specific antiserum to the test *S. typhimurium* LPS resulted in a mean 3 h fever increment of 0.8°C. This represented the lower end of the sensitive portion of the LPS dose-response fever curve; smaller amounts of O-specific antiserum were not tested.[53]

Milner observed minimal protection against LPS-induced fever with rabbit antiserum to an Re chemotype of *S. typhimurium*, and no protection by rabbit antiserum to an Re chemotype of *S. minnesota*, even though LPS from the respective homologous smooth strains were used for challenge.[54] Nor could protection against LPS-induced fever be obtained by Ralovich et al. despite 1 h pre-incubation at 37°C of *E. coli* O83 or *S. enteriditis* LPS with R595 rabbit antiserum, even though the R595 antiserum was capable of markedly inhibiting fever induced by the LPS of the rough mutant R595.[55] In contrast, O-specific antisera in each of the above studies consistently and markedly inhibited production of fever by homologous S-form LPS.

Morris et al. reported that bovine J5 antiserum failed to mitigate the clinical and laboratory alterations that followed sublethal infusions of *E. coli* LPS in neonatal calves.[56] They also reported that equine J5 antiserum failed to protect horses against the cardiopulmonary derangements following challenge with the parental S-form LPS.[57] J5 antiserum did appear to protect sheep against such derangements following challenge with *Serratia marcescens* LPS;[58] however, the J5 antiserum was obtained from donor sheep that had been challenged with *Ser. marcescens* LPS 1 week previously, thereby invalidating the study. Sakulramrung and Domingue reported that J5 rabbit antiserum with high anti-J5 LPS HA titer failed to protect mice against lethal challenge with various quantities of *S. typhimurium* endotoxin; in contrast, antiserum to homologous *S. typhimurium* afforded full protection. The authors concluded: 'we do not have a plausible explanation for the failure of our antiserum to strain J5 to protect mice against lethal doses of endotoxin.'[50] We suggest that these findings reflect the inability of antibodies to J5 or R595 epitopes to bind effectively to S-form LPS (see below).

Evidence against broad-spectrum binding by J5 or R595 antisera to LPS of wild-type smooth enterobacteria

Many in vitro studies support the in vivo observations reviewed above since they demonstrate that murine, rabbit, and human antisera to J5 and R595 are incapable of effective cross-reactivity with S-form LPS from wild-type enterobacteria.

Miner et al. employed a radioimmunoassay (RIA) and observed that murine J5 antisera, although reactive with the J5 and its parental *E. coli* O111:B4 endotoxins, exhibited 'little cross-reactivity with other purified endotoxin preparations'.[59] Using polyclonal J5 rabbit antisera and an immunoblotting technique, de Jongh-Leuvenink et al. noted only weak binding to the parental *E. coli* O111 LPS and no binding to LPS from a heterologous (*S. typhimurium*) wild-type strain.[60] Mehta et al. reported that R595 rabbit antiserum was incapable of binding to the S-form LPS of the parental *S. minnesota* in an RIA.[61]

Cryz et al. demonstrated that murine antisera to J5 LPS failed to cross-react in an ELISA with S-form LPS from three wild-type strains of *P. aeruginosa*.[48] Low titers of antibodies were found to S-form LPS of two other wild-type strains of *P. aeruginosa*, but whether this represented a non-specific polyclonal O-antibody response or cross-reacting antibodies to common LPS epitopes was not determined. McCabe et al. observed no significant changes in HA antibody titers to the S- form LPS of *K. pneumoniae, Morganella morganii*, or *S. typhi* in antisera of rabbits immunized with R595.[29] DeMaria et al. reported similar negative findings in antisera from humans immunized with R595.[62]

Cross et al. examined the cross-reactivity of J5 rabbit antisera with 12 S-form LPS preparations in an ELISA and

concluded: 'thus, the postimmunization lapine antisera had antibody to J5 and lipid A epitopes but...this antibody did not react broadly with the core structures of wild-type strains in this binding assay'.[63] Heumann et al. employed complexes of high density lipoprotein with LPS and its core structures in an ELISA to enhance specificity and sensitivity. They concluded: 'using this technique, it was not possible to find broadly cross-reactive core LPS antibodies after immunization of rabbits and humans with rough mutants of Gram-negative bacteria'. These rough mutants included the R595 and J5 strains. It is particularly pertinent that no cross-reactions were seen with either the IgG or IgM fractions of J5 rabbit or human antisera to the LPS of a variety of smooth enterobacteria including that of the parental smooth *E. coli* O111.[64,65]

The above findings are entirely consistent with those of our own studies[27] and those of Ng et al.,[66] in which R595 or J5 polyclonal rabbit antisera did not agglutinate erythrocytes or bentonite particles coated with LPS from heterologous wild-type enterobacteria. However, even when diluted several 100- or 1000-fold, these antisera agglutinated such particles coated with the respective rough (R) form LPS.

Additional evidence that antisera directed to the LPS core cannot effectively bind in a broad-spectrum fashion to LPS from smooth enterobacteria can be inferred from both competitive inhibition and adsorption studies. HA-inhibition studies by Dlabac, reported as early as 1968, unequivocally demonstrated the ability of O polysaccharides to mask the core antigens in S-form LPS. Red blood cells were coated with LPS from Ra, Rb, or Rc chemotypes of rough *S. typhimurium*. Their agglutination by homologous rabbit antisera could be competitively inhibited by the respective R-form LPS but not by the parental S-form LPS.[67] Masking of core epitopes by O polysaccharides was also evident from competitive-inhibition studies by Schiller, who reported that the bactericidal activity of human serum for a rough strain of *P. aeruginosa* could be markedly inhibited by pre-incubation with the homologous R-form LPS but only minimally by LPS from the corresponding smooth strain.[68]

Schmidt et al. demonstrated that even epitopes of the outer core can mask those of the inner core. Polyclonal antibodies to LPS inner-core epitopes of *E. coli* failed to bind to LPS from rough mutants of the same parental strain containing more complete core structures, as determined by assays of HA and HA inhibition. Similarly, polyclonal antibodies to inner core LPS epitopes of *S. minnesota* that bound to LPS from deep rough *E. coli* mutants could not bind to LPS of *E. coli* rough mutants derived from the same parental strain but containing more complete core structures.[69] This finding was supported by studies of Lyman et al., who demonstrated that bactericidal antibodies in human serum to deep rough mutants of *S. typhimurium* (Rc, Rd1, Rd2, and Re chemotypes) could be adsorbed by these mutants but not by rough *S. typhimurium* mutants containing a more complete LPS core, i.e. Ra and Rb chemotypes.[70] Masking of the inner core by outer core structures was also shown by Lind et al.; three murine MAbs strongly reactive with LPS from Re chemotypes of *Salmonella* were unable to react in ELISA with LPS from Rb1, Rb2, Rb3, Rc, and Rd1 *Salmonella* chemotypes.[71] Subsequent studies by Appelmelk et al. employing ELISA[72] and by Pollack et al. employing both ELISA and passive hemolysis[73] demonstrated that murine MAbs that were highly reactive with LPS from an Rc chemotype of *S. minnesota* could not react with LPS from Ra and Rb *S. minnesota* chemotypes.

However, evidence has now been obtained that outer core structures of LPS do not mask all inner core epitopes: two murine MAbs directed against terminal alpha-pyranoside-linked 2-keto-3-deoxyoctonate (KDO) have been prepared that were capable of reacting by passive hemolysis with all rough chemotypes (Ra–Re) of *S. minnesota*.[74,75] It is important to note, however, that such broad-spectrum cross-reacting antibodies to core epitopes are not present, or present in only small amounts, in polyclonal rabbit antisera to Re mutants of *E. coli*, *S. minnesota*, and *Proteus mirabilis* and that such antibodies do not inhibit S- or R-form LPS-induced fever (E.Th. Rietschel, personal communication).

More recently, evidence has been obtained that O polysaccharides as well as outer-core structures of LPS do not mask all inner core epitopes. Two murine MAbs, isolated after immunization with rough-mutant enterobacteria, exhibited broad-spectrum cross-reactivity with wild-type LPS in vitro (Nnalue et al.,[76] Di Padova et al.[77,78]). The MAb isolated by Di Padova et al. was shown to be pyrogen-free and exhibited broad-spectrum protective activity against pyrogenicity of S-form LPS in rabbits and lethality of S-form LPS in galactosamine-sensitized mice.[77,78] It is unlikely, however, that such antibodies are present in J5 or R595 polyclonal antisera in amounts sufficient to evoke cross-protection for the following reasons: (i) the broadly cross-reactive MAbs were obtained by immunizing mice with rough mutants containing complete (Ra) core epitopes, not with J5 or R595 mutants; (ii) immunization of mice with Rc or Re chemotypes does not evoke such cross-reactive antibodies;[78] (iii) these MAbs were not directed against the Re epitope and would, therefore, not be expected in R595 antisera; (iv) mg/kg amounts of the MAb were required to protect rabbits and mice against ng/kg quantities of LPS; and (v) antibodies cross-reactive with S-form LPS were not identified in J5 and R595 polyclonal rabbit or human antisera by Siber et al.,[31] by Johns et al.,[32] or by Schwartzer et al.[79]

Siber and co-workers critically examined the cross-reactivity of polyclonal IgG and IgM antibodies induced

in rabbits immunized with J5 and R595 rough mutants. High levels of antibodies to the respective J5 or R595 LPS were found in both classes of immunoglobulins, along with relatively low but unequivocal increases in antibody to multiple heterologous S-form LPS. Utilizing ELISA-inhibition, antibody binding to the rough-mutant LPS could be markedly inhibited by the homologous R-form LPS but not by heterologous S-form LPS nor by smooth whole bacteria or their outer membranes. Moreover, antibody binding to the heterologous S-form LPS could be markedly inhibited by the respective S-form LPS but not by other S- or R-form LPS. Siber and co-workers concluded that: 'rabbit antibody directed to J5 or Re595 LPS fails to bind to any substantial degree to heterologous LPS'. They further noted that: 'on the basis of our studies and a review of published reports, we propose that there is insufficient evidence to conclude that the apparent anti-endotoxic activity of antisera to Re595 and J5 mutants is due to antibody to cross-reactive determinants of core LPS... Protection could be due to an increase of modest magnitude but broad spectrum in antibodies to numerous heterologous endotoxins and perhaps to other surface antigens of bacteria.'[31] Earlier observations of Johns et al. are in accordance with these conclusions. They found that R595 rabbit antisera exhibited variable increments in titers of HA antibody to heterologous S-form LPS but that these antibodies appeared to reflect polyclonal B-cell stimulation of O antibodies rather than cross-reactivity, since they could be adsorbed only with the respective heterologous S-form LPS and not with LPS from the R595 mutant.[32] The conclusions of Siber et al. are also in accordance with those of Schwartzer et al. The latter investigators, utilizing a quantitative two-step competitive ELISA, could not demonstrate any cross-reactivity between human polyclonal IgG antibodies to J5 LPS and a panel of enterobacterial S-form LPS, including LPS from the parental *E. coli* strain.[79]

Conclusions based on competitive ELISA data have been criticized by Appelmelk et al., on the grounds that this technique is insensitive for detecting low concentrations of cross-reactive antibodies; i.e. if cross-reactive antibodies comprised only a small proportion of the total population of antibodies to J5 or R595 LPS, their adsorption with heterologous S-form LPS might not appreciably lower the residual titer to the R-form LPS. As evidence, Appelmelk et al. reported that they confirmed the negative competitive-inhibition studies of Siber et al. but that in a direct-binding ELISA, in which Sepharose column affinity-purified antibodies to J5 LPS obtained from polyclonal J5 rabbit antiserum were used, cross-reactivity could be demonstrated against *E. coli, K. pneumoniae, P. aeruginosa,* and their LPS. Since affinity-purified antibodies to J5 LPS were employed, the observed cross-reactivity presumably could not be based on polyclonal O antibodies evoked by J5 immunization.[80] However, these conclusions were in turn criticized by Siber, who stressed that immunoglobulins can be adsorbed nonspecifically to Sepharose columns and that contamination by O antibody had, therefore, not been excluded.[81] We would also stress that the treatment of the smooth Gram-negative bacteria prior to assessment of binding with the affinity-purified antibodies was not specified; as will be discussed, if these organisms had been heat-killed, exposure of rough epitopes may have been artificially induced and could account for the observed binding.

A study by Baumgartner et al. did appear to validate the contention by Appelmelk et al. that adsorption of J5 or R595 antisera with S-form LPS could demonstrate the presence of cross-reactive antibodies to LPS core epitopes if a sufficiently sensitive assay were used. Thus, Baumgartner et al. reported that, if J5 rabbit antiserum were highly diluted (1:10,000), it would exhibit detectable reductions in titer of antibody to J5 LPS after adsorption with whole smooth Gram-negative bacteria, as assessed by ELISA.[82] However, the methodology raises a number of concerns regarding the conclusion that such findings indicate the existence of cross-reactive antibodies to S-form LPS in the J5 antisera. No appreciable reduction in titer of antibody to J5 LPS occurred after adsorption of the highly diluted J5 antiserum with isolated S-form LPS; i.e. the use of whole smooth Gram-negative organisms for adsorption was essential. This finding strongly suggests that the adsorption of antibody to J5 LPS from the highly diluted J5 antisera by whole smooth bacteria might be nonspecific. Nonspecific adsorption, however, was dismissed by Baumgartner et al. because adsorption with a Gram-positive bacterium (*Bacillus subtilis*), with washed packed erythrocytes, or with Sephadex G10 produced no decrease in titer of J5 LPS antibody. We do not agree that these findings exclude nonspecific adsorption of the trace amounts of antibodies to the J5 LPS in the highly diluted antiserum to the rough mutant. Marked reductions of antibodies to *S. minnesota* R595 LPS, which were also present in the J5 antisera, were observed after adsorption with three species of Gram-positive bacteria or with *Candida albicans,* demonstrating that nonspecific adsorption of antibodies to core epitopes can occur. Furthermore, adsorption was carried out with smooth enterobacteria that had been boiled for 2.5 h. No data were provided on the effects of adsorption with non-boiled organisms. As will be discussed, boiling of smooth enterobacteria produces changes in binding characteristics that have no known physiologic relevance; e.g. it exposes rough epitopes and permits binding of antibodies to core glycolipids that otherwise would not occur. More definitive studies on the specificity of adsorption of antibodies to core epitopes from highly diluted J5 antisera by whole smooth enterobacteria, including trials with non-boiled organisms, will be required before the findings

of Baumgartner et al. can be accepted as demonstrating the presence of physiologically meaningful cross-reactive antibodies in J5 antisera.

Finally, converse adsorption studies indicate that no appreciable reductions in antibody titers to various S-form LPS were detectable after adsorption of J5 or R595 antisera with the homologous rough mutants or their LPS, but that the respective S-form LPS markedly reduced these titers.[31,32] These studies demonstrate the serologic preponderance of O-specific antibodies (natural and/or polyclonally-induced) over the putative cross-reactive antibodies in these antisera. Given the proven protective efficacy of O-specific antibodies and the frequency of their polyclonal stimulation with the J5 and R595 mutants (see above), such findings make it particularly crucial that a possible contribution of O-specific antibodies to broad-spectrum protection is excluded before the hypothesis that 'small proportions' of core-reactive antibodies in J5 and R595 antisera are responsible for such protection is accepted. This admonition is identical to that stated by Kenny and Herzberg almost three decades ago: 'experiments involving immunization [of mice] with [*Salmonella*] strains of varying degrees of roughness demonstrated that specific anti-O antibody cannot be eliminated from consideration in protection'.[6]

That the inability of antibodies to inner LPS core epitopes to bind effectively to S-form LPS can indeed entirely account for the ineffective broad-spectrum protection offered by antisera to J5 and R595 rough mutants, and that this is based upon a masking effect of O and outer core polysaccharides was proven in our studies of the ability of rabbit antiserum to the J5 rough mutant to reduce lethality from challenge with J5 LPS. The same pool of J5 antisera that failed to protect mice against the parental S-form LPS was found capable of significantly reducing lethality from challenge with the unencumbered J5 LPS. Moreover, such protection was as marked as that conveyed by O-specific antisera to their homologous S-form LPS.[27] Baumgartner et al. have confirmed these findings in galactosamine-sensitized mice.[43] These findings also parallel those of Ralovich et al. cited previously; i.e. that R595 rabbit antiserum reduced the pyrogenic activity of LPS from strain R595 but not that of LPS from smooth enterobacteria.[55]

Evidence against broad-spectrum binding by J5 and R595 antibodies to non-boiled smooth Gram-negative bact ria

The studies reviewed above demonstrating inability of antisera to J5 and R595 to bind to and reduce lethality from LPS isolated from smooth enterobacteria do not preclude the possibility that effective binding and neutralization might occur when the LPS is displayed in its natural state on the bacterial cell wall. In fact, Nelles and Niswander reported that even though two murine J5 MAbs failed to bind to isolated S-form LPS, including LPS from the parental strain, they did bind to the whole parental organism as well as to many other smooth Gram-negative bacterial strains and species.[83] Similarly, Mehta et al. showed that even though rabbit antisera to R595 and three murine MAbs to R595 failed to bind to the isolated parental S-form LPS, these antibodies did bind to the parental smooth whole organism as well as to heterologous smooth Gram-negative organisms.[61]

Kirkland et al. observed that nine murine MAbs strongly reactive with J5 LPS and its lipid A failed to bind to S-form LPS of *S. minnesota* but could bind to a panel of smooth strains of enterobacteria and Pseudomonas.[84] Pollack et al. reported that although none of four human MAbs to J5 or R595 reacted appreciably with the parental or heterologous isolated S-form LPS, one of these MAbs reacted strongly with heterologous smooth Gram-negative bacteria.[85] Bogard et al. reported that five murine MAbs to J5 or R595 mutants or their LPS that were incapable of binding to S-form LPS (unless these LPS were subjected to SDS-PAGE) could bind to whole smooth Gram-negative bacteria.[86] Miner et al. observed that although J5 murine MAbs showed limited cross-reactive binding with LPS isolated from smooth bacterial strains, binding increased when whole bacteria were employed.[59]

Miner et al. offered several explanations for this important observation. However, the most likely explanation was not considered: the effect of boiling the test bacteria, which was performed in all the above studies. In fact, most studies investigating the usefulness of J5 or R595 antibodies for broad-spectrum protection have considered the binding of antibody to boiled organisms as evidence for broad-spectrum cross-reactivity.[18,32,59,61,82–88] In only one of the above-cited studies did the authors indicate why they boiled the bacteria: the bacteria were boiled for 1 h to remove the capsular antigens.[32] None of the above studies included data on antibody binding to non-boiled bacteria.

When non-boiled bacteria were employed, quite different results were obtained. Gigliotti and Shenep reported that none of four MAbs capable of binding to J5 LPS could bind to non-boiled *E. coli*, including the parental strain.[89] Shenep et al. demonstrated the inability of a J5 MAb to bind to a non-boiled smooth strain of *E. coli* and urged that 'future studies of the binding of antibodies to bacterial cell surfaces avoid non-physiologic treatments of bacterial cells, such as boiling, so as to generate meaningful results'.[90] Even MAbs prepared to the outermost core epitopes of LPS from *E. coli* and *Salmonella* (Ra chemotypes) may fail to bind to the smooth parental strains unless these have been boiled.[91,92] Aydintug et al. have provided additional evidence that boiling alters the reactivity of J5 antibodies with smooth Gram-negative

bacteria. A panel of eight J5 MAbs displayed broad cross-reactivity by suspension ELISA with a variety of smooth bacteria that had been boiled for 2.5 h. In contrast, no broad cross-reactivity was observed when live or formalin-treated bacteria were tested.[93]

Other investigators who used polyvalent R595 or J5 rabbit antisera for analysis of antibody binding to non-boiled wild-type organisms also observed no significant binding to any of eleven serum-resistant strains of *E. coli*[94] or to the parental smooth *E. coli* O111:B4.[95] Overbeek et al. reported that polyvalent rabbit antisera to the J5 mutant reacted only slightly with the viable parental smooth *E. coli* strain.[96]

The effect of boiling could account for the seemingly paradoxical observation that complement-containing J5 and R595 rabbit antisera were strongly opsonic for the homologous rough organisms but could not opsonize diverse wild-type smooth enterobacteria despite their apparent ability to bind to these smooth strains; binding was assessed with boiled organisms, whereas opsonization was assessed with non-boiled organisms.[97] Other investigators also have observed that rabbit and bovine antisera containing high titers of antibodies to *E. coli* J5 or *Salmonella* Re mutants generally are not significantly more opsonic or bactericidal for most viable smooth enterobacteria than are non-immune sera, findings further supporting the ineffectiveness of binding by such polyclonal antibodies to non-boiled enterobacteria.[15,33,39,45,52,97] One study reported that R595 rabbit antisera were more opsonic than normal rabbit sera for non-boiled (but methanol-fixed) *E. coli*. However, this difference did not appear to be related to the presence of cross-reactive core antibodies, since most of the opsonic activity of the R595 antisera remained despite adsorption of all detectable antibodies to the R595 LPS.[94]

Two studies reported significant opsonization of viable smooth organisms by rabbit antisera raised to J5[18] and to R595.[61] However, pre-immune sera from the same donors were not used as controls in these studies; in fact, in one of the studies young (5–6 week old) rabbits were used to provide normal serum whereas adult rabbits were used to obtain the immune sera.[18] Moreover, evidence was not presented to exclude the role of serotype-specific antibodies. This is particularly important in the studies with R595 antisera since bacterial opsonization was tested only against the parental smooth strain.[61] The propensity of rough-mutant enterobacteria to elicit rises in serotype-specific antibodies directed against the parental smooth strain will be emphasized in a later section.

The mechanisms by which boiling permits smooth bacteria to become capable of binding with antibodies to R-form LPS have not been resolved. Heating of wild-type *E. coli* has been shown not only to disrupt the capsule but also to release LPS and to increase surface hydrophobicity, apparently a response to increased exposure of lipid in the outer membrane. These changes resemble those seen in unheated deep-rough mutants of *E. coli* and *Salmonella*.[98] Moreover, Chedid et al. reported that the supernatant from a suspension of smooth *S. typhi* O901 that had been boiled for 2 h contained R-form LPS antigens.[11] Boiling of smooth Gram-negative bacteria thus appears to expose core epitopes on the outer membrane that are otherwise masked. That such a mechanism, rather than heat-induced alterations of S-form LPS *per se* on the bacterial surface, is operative is supported by observations of Nelles and Niswander. They noted that heat treatment of isolated S-form LPS did not expose core determinants, as assessed by direct-binding studies of heat-treated S-form LPS with J5 antibodies and by failure of heat-treated S-form LPS to inhibit binding of J5 antibody to J5 LPS.[83]

Regardless of the underlying mechanism, it is apparent that the use of boiled enterobacteria to prove cross-reactive binding capabilities of antibodies to core LPS epitopes cannot provide valid insights into the potential value of such antibodies for broad-spectrum protection during infection.

It must be noted that Appelmelk et al. reported that some murine J5 MAbs could bind to two non-boiled smooth *E. coli* strains (parental O111:B4 and O125:B15). Compared with polyclonal O-specific antibodies, these MAbs bound 'very much less well', as assessed with immunofluorescence microscopy. In fact, the failure of preincubation with these J5 MAbs to enhance the in vivo clearance of the non-boiled *E. coli* and the inability of these J5 MAbs to protect against the parental *E. coli* in mucin-plus-hemoglobin immunocompromised mice were attributed to the minimal binding of these MAbs to live organisms.[72] In addition to demonstrating such minimal and physiologically nonprotective binding by the murine J5 MAbs to non-boiled smooth *E. coli*, the studies of Appelmelk et al. provide additional evidence against the hypothesis of broad-spectrum protection by antibodies to inner-core LPS epitopes: those J5 MAbs that could bind to the two viable *E. coli* strains failed to bind to any of a panel of other smooth Gram-negative bacteria (*P. aeruginosa, H. influenzae, K. pneumoniae, Pr. mirabilis*, and *Proteus vulgaris*), even after these had been boiled for 2.5 h.[72]

Re-evaluation of the hypothesis that LPS core epitopes become exposed in vivo

In contrast to boiling, Chedid et al. proposed a physiologic mechanism to explain how antibodies to LPS core epitopes might bind during infection: enzymatic factors in serum might unmask rough antigenic sites in the cell wall of smooth bacteria. This might involve direct alteration of S-form LPS or exposure of R-form LPS in the cell

wall.[11] However, Gram-negative bacterial isolates from systemic clinical infections are almost uniformly resistant to the bactericidal action of normal serum,[99] apparently as a consequence of their long-chain O polysaccharide LPS, which appear to prevent insertion of the activated complement C5b-C9 membrane attack complex into the outer bacterial cell membrane.[100,101] In early studies, Dlabac demonstrated that, although smooth strains of *S. typhimurium* were completely resistant to piglet serum, susceptibility increased progressively with an increasing loss of sugar residues from the LPS core.[67] Thus, if normal serum indeed contained enzymes capable of exposing rough core epitopes on cell walls of smooth organisms, all wild-type enterobacteria should be susceptible to attack by complement. In fact, not even the outermost portions of core polysaccharides appear to be unmasked by normal serum, since exposure of the Ra chemotype renders wild-type bacteria susceptible to the bactericidal activity of complement.[102,103]

Furthermore, the serum-enzyme-unmasking hypothesis has been directly tested and not substantiated. Gigliotti and Shenep, using non-boiled organisms, studied binding of four J5 MAbs to the J5 rough mutant and to two strains of smooth *E. coli* (parental O111:B4 and O7:K1), before and after 30 min of incubation in guinea pig serum with complement. Whereas each J5 MAb bound strongly to the J5 mutant, none bound to the non-boiled smooth *E. coli* strains, even after the serum treatment.[89]

Evidence against in vivo exposure of core epitopes by alteration of S-form LPS was elegantly provided by Freudenberg and Galanos. LPS was extracted from the liver of rats 3 days after i.v. injection of *S. abortus-equi* LPS. No exposed rough antigens, whether Ra, Rb, Rc, Rd, Re, or lipid A, were detectable in the recovered LPS, as assessed by competitive inhibition of passive hemolysis; only the original smooth O antigen was evident.[104] These studies extended earlier observations from the same laboratory that found no evidence for alteration of the O-antigenic structure of the LPS in the circulation.[105]

One additional possibility merits special consideration. Antibodies to J5 and R595 core glycolipids might bind to inner core epitopes on smooth enterobacteria during infection as a consequence of epitope exposure during rapid bacterial multiplication or antibiotic therapy. Assessment of the latter possibility is important because Overbeek et al. demonstrated that *E. coli* O111 became more reactive with J5 rabbit antisera when grown for 18 h in Giston broth in the presence of the antibiotic carumonam. They suggested that this antibiotic caused the exposure of LPS core epitopes by interfering with the synthesis of O antigen.[96] Assessment of the effect of bacterial multiplication is important, as indicated by the report of McCallus and Norcross that, in an ELISA, J5 rabbit antiserum reacted with a non-boiled smooth strain of *E. coli* grown in trypticase soy broth for 5 h but was almost unreactive with this strain grown for 19 h. Similar results were seen with the respective *E. coli* LPS extracted with hot phenol-water.[106]

Other possibilities for core epitope exposure during infection include the actions of host defenses on the LPS in the bacterial membrane. For example, Tesh and Morrison observed that the physical characteristics and biologic activity of LPS released from the serum-sensitive J5 rough mutant during incubation with normal human serum differed appreciably from that of the LPS extracted with hot phenol-water and that such differences could not be reproduced simply by the addition of the phenol-water-extracted LPS to serum.[107] Kelly et al. demonstrated decreases in lengths of the O side chain on the LPS of *P. aeruginosa* O1 grown 2–3 days in semipermeable chambers implanted in the peritoneal cavity of rats, as compared with the lengths of the side chains of organisms grown in vitro in proteose-peptone broth.[108]

Our laboratory has not examined the ability of J5 or R595 antisera to bind to or neutralize heterologous wild-type S-form LPS isolated at various periods during in vitro bacterial growth or antibiotic exposure, to LPS released from viable wild-type enterobacteria after incubation with serum, or to LPS released from organisms grown in semipermeable chambers implanted in the peritoneal cavity. We have, however, explored the physiologic implications of such possibilities with respect to the efficacy of J5 and R595 antisera by testing their ability to protect in animal models of sepsis treated or not treated with antibiotics. Rapid bacterial multiplication and/or host modifications of cell wall or of LPS do not appear to be critical factors, in view of the inability of our laboratory, as well as of many others, to demonstrate significant increments in broad-spectrum resistance to viable smooth enterobacteria after active immunization with the J5 or R595 mutants[16,48–52] or passive immunization with antisera raised to these mutants.[16,35–47]

The observations of Sadoff et al. and of Young employing MAbs constitute additional evidence against the hypothesis that antibodies to common LPS core epitopes are protective because of exposure of these epitopes in vivo as a consequence either of bacterial multiplication or of host modifications. Sadoff et al. observed no protection against *P. aeruginosa* in mice passively immunized with a murine MAb to core epitopes of the same strain of *P. aeruginosa* but observed high levels of protection by MAbs directed at the O side chain determinants.[109] Young[110] assessed the ability of murine J5 and R595 MAbs to protect and reported: 'if we use a heavily encapsulated organism, such as an *E. coli* with a K-13 capsule, we have not been able to demonstrate significant protection in an animal given i.v. IgM antibody prior to i.p. challenge. With rough, serum-sensitive organisms, the degree

of protection is considerably greater and statistically significant. These results suggest that in these strains more of the 'core' region of the endotoxin antigen is exposed, and hence can be neutralized by anti-core glycolipid antibodies in the circulation. It is re-emphasized, however, that rough serum-sensitive strains are infrequently encountered in systemic human infections.[99] This is discussed in detail in a later section.

Since neither our laboratory nor those cited above[16,35–47] could demonstrate significant broad-spectrum protection against smooth enterobacteria or *P. aeruginosa* in animals not treated with antibiotic but given polyclonal antisera to core epitopes, we assessed the efficacy of J5 and R595 rabbit antisera given at various intervals during the course of enterobacterial sepsis (*E. coli* O18, *Pr. mirabilis*, and *K. pneumoniae*) in mice treated with antibiotics. Aminoglycosides, to which these bacilli were highly sensitive, were found capable of shifting the lethal mechanisms during infection from those involving bacterial proliferation and low levels of endotoxemia to those involving bacterial death and release of large amounts of circulating endotoxin.[111] Such released endotoxin appeared to be a critical factor, since mortality correlated with the increments in plasma endotoxin and since adrenal glucocorticosteroids (which were nonprotective in the absence of antibiotics) now became highly protective.[36,111] However, in these models of Gram-negative bacterial sepsis treated with aminoglycosides, just as in those not treated with antibiotics, J5 and R595 rabbit antisera remained unable to reduce mortality, even though testing was performed within sensitive dose-mortality ranges; in contrast, O-specific antisera remained protective.[36] Thus, these findings do not support the hypothesis that anti-core antibodies in J5 or R595 antisera become protective in vivo as a consequence of exposure of inner-core epitopes during bacterial multiplication, host-defense processing, and/or aminoglycoside antibiotic therapy.

A report by Flynn et al. should be addressed at this point since it bears directly upon these considerations. A J5 murine MAb (M1B1) which could not bind to *E. coli* O7:K1 did so after incubation with the antibiotic moxalactam for 15–60 min. The authors concluded: 'these findings suggest that the core glycolipid regions of the lipopolysaccharides of some Gram-negative bacilli can be effectively sequestered until liberated by antibiotic cell lysis'.[112] This conclusion, however, should not be interpreted as indicating that LPS in smooth Gram-negative bacteria may be altered by antibiotics so as to expose cryptic core glycolipid epitopes. We have reviewed the extensive evidence that J5 polyclonal and monoclonal antibodies to core epitopes of LPS are incapable of effectively binding to isolated S-form LPS. The demonstrated binding of M1B1 to the phenol-water extracted LPS of the *E. coli* O7:K1 strain used by Flynn et al. suggests that this LPS differs from most other S-form LPS. That the *E. coli* O7:K1 LPS, rather than M1B1, was unique is indicated by the observation that M1B1 could not bind to the isolated LPS of the parental *E. coli* O111:B4. We, therefore, propose that the findings of Flynn et al. are most readily explicable by observations of Cross et al. The latter group, employing rough-specific phages, demonstrated that bacteremic isolates of K1-encapsulated *E. coli* often possessed rough or semi-rough LPS in their outer membrane, and that the capsule masked their otherwise exquisite sensitivity to serum bactericidal activity.[113] Binding of M1B1 after moxalactam treatment could occur simply because of the resulting capsular disruption. Polyclonal antibodies raised to decapsulated *E. coli* O7:K1 have been shown in earlier studies to also be incapable of binding to the intact organism until the capsule was disrupted by heating.[44]

We are unaware of studies that have critically explored the capability of J5 or R595 antisera to protect against capsular-masked phenotypically rough *E. coli*, or to enhance their protective activity against such strains when given together with capsular-disruptive antibiotics. This will be considered further in a later section.

Re-evaluation of the hypothesis that IL-2 evokes broad-spectrum protection against Gram-negative bacterial infection by inducing polyclonal antibodies to J5 LPS

In 1986, Weyand et al. observed that recombinant IL-2 (rIL-2) evoked polyclonal IgM antibody responses in mice and subsequently tested rIL-2 for its protective activity against *E. coli* sepsis. Pretreatment with rIL-2 significantly reduced mortality following i.p. challenge of female BALB/c mice with *E. coli* O4:K6:H+. Protection could be passively transferred by IgM serum fractions and was associated with increments in IgM antibodies to J5 LPS, as well as to numerous other antigens. Comparative studies employing active immunization with the J5 mutant were also performed. The following conclusions were drawn with respect to the role of J5 LPS antibodies: 'although the mechanism of protection [by rIL-2] has not been completely clarified, there is a striking correlation between the ability of J5 antigen or rIL-2 to induce IgM antibodies specific for the LPS core J5 antigen and the protective efficacy of both agents'.[114] However, these inferences regarding the efficacy of J5 antibodies and their role in protection by rIL-2 do not appear justified. No assessment was made as to whether the polyclonal antibodies evoked by rIL-2 included those with capsular or O-specificity for the *E. coli* challenge strain. This is a critical issue since, as reviewed above, such antibodies have high protective activity and since other polyclonal stimulating agents such as rough enterobacterial mutants have been shown capable of evoking polyclonal O-specific antibodies to multiple serotypes of smooth *E.*

coli.[31,32] Furthermore, immunization of mice with the J5 vaccine did not provide significant protection against mortality from the challenge *E. coli* strain despite eliciting ELISA antibody titers to J5 LPS approximately 50% higher than those induced by the protective rIL-2 pretreatment.[114]

Re-evaluation of the basis for the requirement of compromised host defenses for broad-spectrum protection by J5 and R595 antisera

It has become increasingly apparent that to demonstrate protection against enterobacterial infection with J5 and R595 antisera, it may be necessary to employ animal models that use resistance-lowering agents such as nitrogen mustard, or hog gastric mucin and/or hemoglobin,[13–15,21,50,115] all of which greatly enhance mortality and permit marked reductions in the size of the challenge inoculum. For example, Young and Stevens indicated that the protection afforded by R595 rabbit and canine antisera in mice challenged with *E. coli* was 'critically dependent' upon the concentration of hog gastric mucin administered concomitantly.[115] Sakulramrung and Dominque reported that no protection was afforded by high-titer J5 rabbit antiserum given to mice 1 h before challenge with 2 LD_{50} of *K. pneumoniae*, *E. coli* O6, or *E. coli* O4, or even with *K. pneumoniae* that had been preincubated with the antiserum,[50] confirming our and other investigators' negative findings.[35–47] This antiserum, however, protected mice against challenge with viable *E. coli* O4 when sheep hemoglobin was administered concomitantly.[50] Appelmelk et al. demonstrated that J5 rabbit antisera could not protect normal mice against challenge with the parental smooth *E. coli* (again confirming the previous negative findings), but could protect if the mice were given mucin plus hemoglobin.[21]

Appelmelk et al. hypothesized that immunocompromising agents are required to demonstrate protection by antisera to rough mutants because antibodies to core LPS epitopes are relatively ineffective compared with antibodies to O-specific antigens; but these antibodies become protective against challenge with the smaller inocula used in the compromised host because of the greater amounts of antibodies per organism.[21] However, we would stress that the same mechanism would also magnify the protective activity of O-specific antibodies. As reviewed above, antisera to rough mutants often contain polyclonal increments in O-specific antibodies. Indeed, J5 and R595 rough mutants were shown by Siber et al. not only to evoke polyclonal IgG and IgM serotype-specific antibodies to a variety of S-form LPS but also to do so much more effectively than wild-type enterobacteria.[31] This accords with earlier observations by Bruins et al. that antibody titers to heterologous S-form LPS were higher in rabbits immunized with Re Salmonella mutants than in rabbits immunized with *P. aeruginosa*,[17] and with the reported propensity of R595 LPS to induce markedly greater increments in polyclonal IgG and IgM antibody production in mice than S-form LPS.[116] Appelmelk et al. provided no data with respect to serotype-specific antibodies in the protective J5 polyclonal rabbit antisera given to immunocompromised mice.[21] Such data would be particularly critical in these studies, since only protection against the parental smooth strain was examined; J5 antisera (as well as R595 antisera) often contain especially marked increments in serotype-specific antibodies to the parental smooth strain.[24,27,31–33,53,59,60]

The J5 rabbit antiserum used by Ziegler et al. to demonstrate protection in immunocompromised rabbits against oral challenge with *E. coli* O4, *E. coli* O17, and *K. pneumoniae* had HA titers of 1:16 for *E. coli* O4 LPS and 1:512 for *E. coli* O17 LPS (HA titers to *K. pneumoniae* LPS were not specified). The control non-immune sera had titers of 1:4 to *E. coli* O4 LPS and 1:16 to *E. coli* O17 LPS. No evidence was provided that the increments in titers did not reflect rises in O-specific antibodies, which could account for the observed protection.[13] Moreover, since pre-immune sera from the same serum donors were not specified as controls, the contribution of natural O-specific antibodies (or other serum factors) to the protection by the J5 antiserum cannot be excluded. The same investigators reported that J5 rabbit antiserum reduced mortality in granulocytopenic rabbits challenged by intraconjunctival instillation of *P. aeruginosa*.[14] However, the same reservations apply to this study. Similar reservations also apply to the protection reported by Sakulramrung and Domingue with J5 polyclonal rabbit antiserum in immunocompromised mice challenged i.p. with *E. coli* O4 mixed with hemoglobin.[50]

The possibility that polyclonal increments in O antibodies, rather than antibodies to LPS inner core, become protective when host resistance and size of challenge inoculum are reduced is supported by the findings of Appelmelk et al. that, in contrast to the protection offered by polyclonal J5 rabbit antisera, a variety of murine J5 MAbs – either individually or pooled – did not protect against the parental smooth *E. coli* strain in mucin- and hemoglobin-compromised mice.[72] In addition, three other groups of investigators were unable to demonstrate broad-spectrum protection by polyclonal rabbit or human J5 antisera in immunocompromised mice.[41,43,117]

It is emphasized that the protective activity of O-specific antibodies can be so marked that, even in the non-immunocompromised host, an apparent absence of O antibodies in rough-mutant antisera may not exclude their role in protection. In 1968, Kenny and Herzberg studied protection against smooth enterobacteria evoked in mice by *Salmonella* rough-mutant vaccines. They

emphasized that other investigators had reported that protection could be induced in the absence of O agglutinins, and that this implied that O antibody is not important in protection. Using a more sensitive bactericidal assay, however, they reported: 'results of this investigation showed that the presence of the antibody response was demonstrable and that it was specific for the smooth homologous [parental] strain'.[6]

Two decades later, the importance of the sensitivity of the assay system for O-antibodies in elucidating the mechanism of protection by rough-mutant antisera was elegantly re-affirmed by Saxen et al. Rabbit antisera to a complex of purified porin and R-form LPS (both extracted from an Rb rough mutant of *S. typhimurium*), possessing no detectable O antibodies by ELISA, were found to protect mice from *S. typhimurium* mortality by mechanisms that could only be accounted for by O-specific antibodies: high levels of protection were conferred against mortality from *S. typhimurium* O-4,12 but no protection was found against isogenic *S. typhimurium* transductants whose O antigen was genetically switched to O-9,12. Saxen et al. concluded that, in their model, mouse protection constitutes one of the most sensitive assays for anti-O antibodies.[10]

In view of the above, it is not surprising that immunocompromised mice have been shown to be protected against *E. coli* bacteremia by amounts of O-specific antibodies undetectable by assays as sensitive as ELISA.[118] More recently, male mice with an X-linked immunodeficiency, lacking natural O-specific antibodies to *P. aeruginosa* immunotypes 1–7, were found to be no more sensitive to challenge with *P. aeruginosa* than were their female cohorts who possessed low titers of such antibodies. However, when animals of both sexes were immunocompromised with cyclophosphamide, the males became 50–1000 times more sensitive to the lethal effects of *P. aeruginosa* challenge and resistance could be restored by passive transfer of as little as 0.2 μg of O-specific MAbs.[119,120]

It is apparent that the use of immunosuppressed hosts to demonstrate broad-spectrum protection by cross-reactive antibodies to LPS core epitopes in rough-mutant antisera mandates inordinate precautions to exclude the role of natural and induced polyclonal O antibodies.

Immunosuppression has not always been a prerequisite for demonstrations of broad-spectrum protection by antisera to rough mutants. Four successful studies in animals with intact defenses have been reported.[11,12,18,29] In three of these studies,[11,12,29] mice were challenged with the highly virulent Caroli strain of *K. pneumoniae* type II. McCabe et al. speculated that the use of this organism circumvented the need for immunosuppression by permitting the use of inocula small enough to make multiple cycles of replication necessary for lethal infections; presumably, antibodies to inner core LPS epitopes would bind more readily to the bacilli during each growth phase, when binding reactivity may be greater. The failure of other investigators to demonstrate broad-spectrum protection by J5 and R595 antisera in non-immunosuppressed animals was attributed to the fact that 'large inocula ($> 10^7$) have been used almost exclusively in those investigations that have been unable to demonstrate such protective activity'.[29]

Large inoculum size, however, cannot satisfactorily explain the failure to demonstrate protective activity of J5 and R595 antisera. The original report by McCabe on cross-protection by R595 rabbit antiserum was based upon challenge of non-immunocompromised mice with two smooth organisms, the Caroli strain of *K. pneumoniae* and *E. coli* 107. Highly significant protection was observed against challenge with 10^8 *E. coli* 107,[12] even though the HA antibody titers to R595 LPS were no greater, or considerably lower, than in studies that failed to demonstrate protection against other *E. coli* strains.[35,44] We re-examined the ability of rabbit antisera to J5 and R595 rough mutants to protect mice against the same small inocula (10^4 cfu) of the same Caroli strain of *K. pneumoniae* used by McCabe. We confirmed the observation that challenge with 10 bacilli produces 100% mortality and that only the rate of death was influenced by the inoculum size. With 10^4 cfu organisms, mortality approached 100% by the 5th day. We could observe no differences in mortality at any time despite pretreatment with J5 antiserum or with R595 antiserum possessing HA titers to R-form LPS comparable to that employed by McCabe as long as comparisons were made with matched pre-immune sera.[35] Trautmann and Hahn confirmed these negative findings using the Caroli strain and J5 rabbit antiserum.[40] Ng et al. also were unable to demonstrate protection with R595 rabbit antisera in mice challenged with even smaller inocula (1.5×10^3 cfu) of the Caroli strain.[37] Nor could Baumgartner et al. demonstrate protection against *E. coli* O111 or *P. aeruginosa* serotype 3 infection in mucin-plus-hemoglobin compromised mice by pretreatment with rabbit J5 antiserum even though the LD_{50} was 1.7×10^5 cfu for *E. coli* and only 5 for the *Pseudomonas* strain.[43] Vuopio-Varkila failed to observe protection by J5 rabbit antiserum in cyclophosphamide immunocompromised mice against challenge with only 2×10^4 *E. coli* O18:K1.[41]

As with infection models, it has been implied by Ziegler that impairment of host defenses with an agent such as actinomycin D may be required in order to discern broad-spectrum protective activity of J5 and R595 antisera against lethality from S-form LPS. The marked decrease in the challenge dose of LPS afforded by sensitization with actinomycin D presumably would allow detection of such protective activity.[121] However, this

contention was not supported by earlier studies of Johns et al., in which protective activity of R595 rabbit antisera against wild-type LPS was as readily detected in mice with and without actinomycin D.[24] Our experiments, when repeated with actinomycin D-sensitized mice, again failed to detect protective activity of R595 or J5 rabbit antisera against challenge with S-form LPS.[27] Studies by Baumgartner et al. also demonstrated that J5 rabbit antiserum failed to provide significant protection against LPS from the parental smooth *E. coli* O111 in galactosamine-sensitized mice.[43]

We conclude that, in those studies in which challenge with small quantities of Gram-negative bacteria or their LPS is required for demonstration of broad-spectrum protection by J5 or R595 antisera, such protection should not be presumed to be mediated by core antibodies until other serum factors with known protective activity have been excluded. These include natural and polyclonally-induced O-specific antibodies and acute-phase serum reactants. The latter will now be considered.

Mechanisms not mediated by antibody that may underlie broad-spectrum protection by antisera to rough mutants

The possibility that non-immune mechanisms can protect against the adverse effects of S-form LPS was proposed more than three decades ago by Raskova: 'our experiments indicate that light irritation of tissue by simple chemical compounds (phenol, procain) can mimic, in many ways, endotoxin-induced tolerance'. Such nonspecific tolerance was transient (waning markedly within 4 weeks) and could be passively transferred with serum.[122] Evidence has since accumulated that acute phase serum proteins can mediate such nonspecific protection against endotoxin as well as against Gram-negative bacterial sepsis. Kindmark reported that highly purified human C-reactive protein (CRP), an acute phase protein, acted as an opsonin for both Gram-positive and Gram-negative bacteria when assessed in vitro with human peripheral blood leukocytes; the Gram-negative bacteria included three strains each of *E. coli* and *Klebsiella aerogenes*, and in each case opsonization was highly significant ($P < 0.001$). Kindmark concluded: 'CRP may combine with the bacteria and thereby act as an opsonin with consequent promotion of the non-specific resistance to infections'.[123] van Vugt et al. demonstrated that rat α_2-macroglobulin, another acute phase protein, protected rats against the early phase of endotoxin-induced shock.[124] Warren et al. reported that rabbit serum obtained 24 h after the last of six daily injections of LPS from *E. coli* O18 neutralized greater amounts of heterologous S-form LPS than did pre-immune sera, as assessed by the *Limulus* amebocyte lysate (LAL) gelation assay.[125] Warren et al. subsequently demonstrated that rabbit sera obtained 7 days after a 3 week course of immunization with boiled *E. coli* O113, *E. coli* O18, *E. coli* J5, or *S. minnesota* R595 also neutralized increased amounts of heterologous S-form LPS as assessed by the LAL gelation assay. Of especial importance was the finding that, although the antisera to the J5 and R595 rough mutants proved more potent than the antisera to the wild-type *E. coli* O113 and *E. coli* O18 in neutralizing heterologous S-form LPS, the enhanced broad-spectrum neutralizing activity of the J5 antiserum could not be related to IgG or IgM antibodies to the rough-mutant LPS.[126]

The observation that LPS incubated in samples of fresh frozen human plasma with high endotoxin-neutralizing activity, as determined by the LAL assay, induced less fever in rabbits than did LPS incubated in plasma samples with low neutralizing activity suggested that the LAL endotoxin-neutralization assay may have physiologic relevance. The additional observation that plasma pools with high and low LPS-neutralizing activity possessed similar amounts of serotype-specific, J5, and R595 LPS antibodies in both IgG and IgM classes suggested that such antibodies were not responsible for the differences in activity.[127] Moreover, LPS-neutralizing activity was significantly increased in sera from all groups of patients with inflammatory diseases, including a group with Gram-positive bacteremia.[128]

Studies by Riveau et al.[129] and by Warren et al.[130] subsequently implicated interleukin-1 (IL-1) as one of the mediators produced during immunization with Gram-negative bacteria or LPS that accounts for the enhanced ability of serum to neutralize heterologous LPS. IL-1 appeared to act by inducing the production of acute-phase proteins that enhance the binding of LPS to serum lipoproteins.[130] It has since been shown by Wurfel et al., that the acute phase protein, lipopolysaccharide-binding protein (LBP), can transfer LPS to circulating lipoproteins and thereby neutralize LPS bioactivity; however, it can also facilitate the interaction of LPS with CD14 cell receptors on macrophages and thereby enhance the toxic activities of LPS. The authors conclude: 'this dual role of LBP...makes it difficult to predict whether its effects are predominantly to enhance or blunt responses to LPS'.[131] Studies by Gallay et al. suggest that the former effects predominate since IgG antibody to murine LBP protected mice against LPS lethality.[132,133] However, demonstrating the protective effects of anti-LBP and the precise role of LBP in the non-specific protection induced by substances such as turpentine and by IL-1 will require further study.

That acute phase proteins are important in protection against LPS and Gram-negative bacterial sepsis is supported by two recent studies using D-galactosamine. Alcorn et al. presented evidence that the remarkable increase in sensitivity to LPS lethality induced by D-galactosamine

results from inhibition of the hepatic synthesis of acute phase proteins. Pre-activation of acute phase protein synthesis by turpentine given subcutaneously 24 h beforehand prevented the sensitization.[134] Vogels et al. concluded that activation of acute phase hepatic protein synthesis by IL-1 was the primary mechanism by which this cytokine protected mice against *P. aeruginosa* and *K. pneumoniae* sepsis, since inhibition of acute phase protein synthesis with D-galactosamine abolished the protective activity of IL-1.[135]

The concept, that non-specific protection against Gram-negative sepsis and LPS elicited by pretreatment with various chemical compounds and with IL-1 is based upon their ability to induce acute phase proteins, requires reconciliation with the ineffective protective activity of another interleukin, IL-6, capable of inducing acute phase proteins. In 1989, van der Meer et al. reported that pretreatment (t = −24 h) of granulocytopenic mice with recombinant human IL-6 (rhIL-6) in amounts up to 800 ng failed to significantly protect mice against i.m. challenge with *P. aeruginosa*, or potentiate the protective effect of IL-1 pretreatment against such challenge.[136] In 1993, van der Meer's laboratory extended these findings, demonstrating that pretreatment (t = −24 h) of granulocytopenic mice with up to 8000 ng rhIL-6 again failed to provide protection against i.m. challenge with *P. aeruginosa*. Recombinant murine IL-6 (rmIL-6) was also ineffective in amounts up to 1600 ng.[135] In 1993, Barton and Jackson reported that rIL-6 given at t = −1 h conferred no protection to mice challenged i.p. with LPS combined with D-galactosamine.[137] In all of these studies, however, the bioactivity of IL-6 for the induction of acute phase proteins was not assessed. Evidence of such bioactivity is required before concluding that IL-6 is not protective.

Studies from Morrison's laboratory provide the most definitive demonstration that the acute phase response, as induced by IL-6, does not protect mice against LPS. These studies utilized both normal and D-galactosamine sensitized mice challenged with LPS at various times (3, 6, 12, 14 h) after pretreatment with varying amounts of rmIL-6. The IL-6 was shown to elicit an acute phase response that was evident at 6 h and maximal after 10 h as indicated by rises in circulating fibrinogen and C3. The IL-6 failed to protect mice against mortality following i.p. LPS challenge or against LPS combined with D-galactosamine, nor did it prolong survival time in either group. In addition, there was no difference between the effects of IL-6 induced acute phase murine serum and normal serum on LPS-stimulated TNFα release from thioglycolate-elicited murine peritoneal macrophages.[138]

Vogels et al.[135] proposed an hypothesis to account for this dichotomy in protection by IL-1 and IL-6: 'the fact that IL-6 as an inducer of acute phase proteins is not capable of enhancing nonspecific resistance, whereas acute-phase proteins themselves seem to do so, is not necessarily contradictory. IL-6 and IL-1 are known to induce different spectra of acute-phase proteins, and it is conceivable that the IL-1 induced spectrum is protective and the IL-6 induced spectrum is not.' Bucklin et al. had previously suggested a similar possibility: 'one interpretation of the [negative] results obtained in these studies would be that mrIL-6, although clearly capable of inducing increased levels of fibrinogen or C3, does not induce the production of LPS-binding proteins'.[138]

Studies in IL-6 gene knockout mice have helped to delineate the role of IL-6 in the acute phase response to various stimuli.[139,140] In these mice, the elicitation of various acute phase proteins was severely compromised after tissue damage with turpentine or Gram-positive infection with *Listeria monocytogenes*, whereas considerably smaller reductions were seen in response to LPS. It was concluded:[139] 'thus, IL-6 is an important mediator of an acute-phase response after tissue damage or infection with Gram-positive intracellular bacteria but not Gram-negative bacteria. Bacterial LPS...triggers inflammatory mediators from a variety of cell types and may therefore be less dependent on IL-6.'[1]

Since inflammatory mediators such as IL-1 significantly enhance resistance to Gram-negative bacterial infections and LPS,[129,130,135,136,141-143] it is imperative that any study designed to explore the mechanism of broad-spectrum protection induced by rough-mutant vaccines evaluate the contribution of inflammatory responses which such vaccines provoke. It would be necessary, therefore, in studies claiming broad-spectrum protection by vaccines containing R-form LPS, to employ controls that possess comparable inflammatory activity, e.g. pyrogenicity, and that induce comparable increments in selected acute phase proteins, e.g. LPS-binding proteins, before concluding that antibodies to common core epitopes are responsible for protection.

The persistence of broad protection for long periods after completion of immunization may not exclude participation of acute phase reactants. Koh stated: 'individual acute phase proteins show different patterns of return to normal: haptoglobin and α_1-antitrypsin are rather fast in this respect, while increased levels of fibrinogen and orosomucoid may persist in the blood for a long time, even in the absence of complications'.[144] Repetitive injections of inflammatory stimuli appreciably alter the patterns of acute phase protein responses.[145,146] Baumann and Gauldie emphasized that, while the acute phase reaction usually subsides rapidly, this can be prolonged by persistence of stimulation or by disruption of normal control mechanisms and can convert to a chronic phase.[147] We are unaware of studies of the length of persistence of various acute phase serum reactants, in particular LPS-binding proteins, after the multiple

inoculations of LPS-containing vaccines routinely used to induce broad-spectrum protection. Most studies of broad-spectrum protection by rough-mutant antisera have been conducted with sera drawn 1 week after completion of a series of inoculations with boiled rough-mutant organisms. We are also unaware of studies of the comparative effects of S and R-form LPS vaccines on the intensity, duration, and pattern of the acute phase response. Because marked differences exist in distribution and degradation of S and R-form LPS,[148,149] even the use of equally inflammatory amounts of these vaccines may not ensure comparable acute phase responses. Finally, it should be noted that since the pattern of hepatic acute phase protein response to the same cytokine is variable between species,[150] the protection resulting from acute phase responses to LPS vaccines in one species cannot *a priori* be extrapolated to another.

We conclude that the contribution of acute phase serum factors must be carefully excluded before broad-spectrum protection induced by vaccination with rough enterobacterial mutants or by antisera raised against such mutants is attributed to antibodies to core LPS epitopes. We recognize that accurate assessment of the role of acute phase serum factors is at present extremely difficult. Conclusions based upon studies with rough and smooth vaccines possessing equally potent inflammatory properties may not be valid because of the differences in distribution of S- and R-form LPS. Unfortunately, studies in animals cannot be extrapolated to humans, and definitive exclusion of acute phase reactants must await further identification of the protective factors.

R -evaluation of the significance of loss of broad-spectrum protection by J5 and R595 antisera after adsorption with R-form LPS

Some investigators have attempted to prove that the broad-spectrum protection by J5 and R595 antisera observed in mice was indeed related to antibodies to LPS core by showing that this activity was lost after the antisera were adsorbed with R-form LPS.[11,15,19,20,24,50] However, as emphasized by Siber and co-workers,[31] the technical difficulties involved, particularly the problem of contamination of the antiserum with the LPS used for adsorption, make it extremely difficult to exclude the role of serotype-specific antibodies, antibodies to bacterial antigens other than LPS, or non-antibody factors.

For example, the protective activity of J5 rabbit antiserum against mortality of mice challenged with *H. influenzae* type b was completely abrogated, and in fact mortality was enhanced, after adsorption of the antiserum with formalin-treated human erythrocytes coated with J5 LPS. The adsorption was found to contaminate the serum with LPS, so that 1 ml given i.v. to rabbits produced a 'pronounced' pyrogenic response (3 h increments of 4°C), whereas the unadsorbed serum was non-pyrogenic. The authors stated: 'the fact that there were no survivors in the adsorbed J5 antiserum group suggests that contaminating LPS may have exerted a toxic effect'.[20] In a later study, the J5 antiserum was found to be incapable of cross-reacting in ELISA with *H. influenzae* type b LPS or of neutralizing its toxicity. A nonspecific serum factor induced by immunization was proposed as a possible mediator of the observed protection.[151]

Another example is the report by Johns et al. that adsorption of R595 rabbit antiserum with human erythrocytes coated with R595 LPS abrogated its protective activity in mice against lethality from S-form LPS of *S. typhi* and *S. minnesota*. In contrast, adsorption of the R595 antiserum with erythrocytes coated with the respective S-form LPS failed to reduce the protective activity of the antiserum. LPS contamination did not appear sufficient to account for these results.[24] These findings indicate that the putative cross-reactive antibodies in the R595 antiserum do not bind to S-form LPS in vitro, and that protection by these antibodies must, therefore, occur after the S-form LPS has been altered in vivo, exposing R-form epitopes. However, as reviewed earlier, direct evidence does not support such in vivo alteration.[104,105] Moreover, if this did occur, S-form LPS would be expected to induce antibodies cross-reactive with R595 LPS. This has not, however, been observed.[31–34]

A third example is the report by Konstantinov et al. who employed adsorption studies to demonstrate that protection of mice against *S. typhimurium*, *E. coli*, and *P. aeruginosa* by rabbit antiserum to an Rc mutant of *S. minnesota* was mediated by antibodies to the Rc core epitopes.[19] No measurements of O antibodies were made. Rather, it was assumed that antibodies to Rc epitopes were responsible for protection because repetitive adsorptions of the antiserum with the heat-killed Rc mutant abrogated protection against *S. typhimurium*. However, it is unlikely that antibodies to the Rc LPS were responsible for protection; McCabe observed that rabbit antiserum raised against the same Rc mutant, possessing comparable HA titers to the Rc LPS, did not confer protection to mice against comparable sized inocula of *E. coli*.[12] Since no mention was made of washing the bacteria used for adsorption or assessment of the resultant LPS content of the antiserum, it is likely that endotoxin contamination, rather than adsorption of antibodies to the Rc epitopes, was responsible for this loss of protection.

A fourth example is the report by Chedid et al. that an equine antiserum to an Ra rough mutant of *S. typhimurium* protected mice against lethality from the Caroli strain of *K. pneumoniae*. Protection was abolished by adsorption with several heat-killed rough strains of *Salmonella* but not by adsorption with several smooth

strains. These observations formed the basis for the hypothesis that rough-mutant enterobacterial vaccines can evoke antibodies to common core epitopes which can provide broad protection against smooth enterobacteria once their core epitopes are unmasked in vivo by host enzymes.[11] However, two important findings regarding adsorption were not accounted for:

1. In contrast to the inability of the smooth strains of *Salmonella* to adsorb protective activity from the Ra antiserum, adsorption with the smooth challenge strain of *K. pneumoniae* abolished protection. Such specificity suggests the role of specific, not cross-reactive, antibodies. Since pre-immune serum controls were not included, these protective specific antibodies may have been naturally occurring and need not have been polyclonally induced by immunization with the Ra mutant. We observed that all normal equine sera tested protected mice against lethality of the Caroli strain and that such protection could be abolished by adsorption with *K. pneumoniae* but not by a heterologous smooth enterobacterium. Furthermore, we found that normal equine sera contain antibodies to Ra (*Salmonella*) and Rc (J5) epitopes, and that the titers of these antibodies were unchanged in the sera adsorbed with *K. pneumoniae* and no longer protective.[35]

2. Loss of protection after adsorption with rough *Salmonella* strains was variable; some rough strains possessing the same chemotypes did not remove protective activity. For example, adsorption with heat-killed R595 failed to remove any protective activity, whereas adsorption with another Re *Salmonella* mutant (G30/C21) totally ablated protection. Variable levels of endotoxin contamination, which were not assessed, rather than adsorption of antibodies to LPS core determinants, would explain these findings, particularly since trace amounts of LPS have a marked influence on the lethality of the Caroli strain in mice.[152]

We conclude that the available adsorption data do not provide convincing evidence for the existence of cross-protective antibodies to LPS core epitopes in rough-mutant antisera.

Evidence for a limited spectrum of protection by J5 and R595 antisera

As reviewed above, antibodies to core epitopes in antisera to J5 and R595 appear incapable of broad-spectrum neutralization of smooth enterobacterial LPS or protection against wild-type Gram-negative bacteria. Nevertheless, examples of selective protection by polyclonal antisera to Rc and Re LPS core epitopes can be cited. Cryz et al. observed that mice immunized with LPS from an Rc chemotype of *Salmonella* (*S. typhi* Ty21a) or with J5 LPS developed IgG antibodies selectively reactive in ELISA with LPS from a serotype 3 strain of *P. aeruginosa*; no appreciable binding to LPS from strains of serotypes 1, 5, 6, or 7 was seen. In a burn wound sepsis model, the immunized mice were found to be protected against challenge with viable *P. aeruginosa* serotype 3 but not against the other four smooth strains. Whether the protective antibodies were directed to core epitopes of the rough-mutant LPS vaccine and cross-reactive with the LPS of *P. aeruginosa* serotype 3 or were polyclonally induced antibodies specific for serotype 3 LPS was not determined.[48] Nys et al. reported that J5 and R595 rabbit antisera protected actinomycin D-sensitized mice against lethality from *E. coli* O7 LPS, but not from *S. enteriditis* LPS.[153] Whether this represents another example of selective protection or is relatable to differences in titers of O antibodies was not explored.

Bhattacharjee et al. reported protection of immunocompromised rats against *P. aeruginosa* serotype 6 by J5 rabbit antiserum and by sepharose column preparations of anti-J5 IgG as well as affinity-purified anti-J5 IgG.[154,155] Since Baumgartner et al. observed no protection by J5 rabbit antisera against *P. aeruginosa* serotype 3 in immunocompromised mice,[43] it is likely that the protection against serotype 6 represented selective, not broad-spectrum protection. Nevertheless, broad-spectrum protection was suggested by Bhattacharjee et al. based upon subsequent in vitro studies indicating that an anti-J5 IgG fraction from a single rabbit bound in a flow cytometric assay to a greater percentage of viable organisms from several strains of *E. coli*, *P. aeruginosa*, and *Enterobacter*, and one strain of *K. pneumoniae* compared to pre-immune IgG. However, the increments in binding were highly variable; almost half of the bacterial strains exhibited negligible increments in binding and inexplicably, the J5 IgG fraction bound to 11.6% of viable J5 organisms, in contrast to 44.3% of the *P. aeruginosa* serotype 6 challenge strain.[155]

Davis et al. reported that J5 rabbit antiserum was capable of binding to LPS from group A, B, and C meningococci, as assessed by an HA assay.[28] Meningococcal (as well as other neisserial) LPS do not possess O polysaccharide side chains. Rather, they are comparable to the unencumbered R-form LPS in rough-mutant enterobacteria and possess both group-specific and more broadly reactive epitopes.[156–158] Since they lack covering O polysaccharides and since J5 antisera can protect against unencumbered R-form LPS,[27,43,55,59] it would not be surprising if J5 antisera that possessed cross-reactive antibodies to common epitopes in meningococcal LPS could protect against the isolated LPS. This appears to be the case, as evidenced by the suppression of meningococcal LPS-induced dermal and generalized Shwartzman reactions

by J5 rabbit antisera. The evidence is incomplete, however, since there was no correlation between HA titers to meningococcal LPS in the J5 antisera and anti-Shwartzman activity and since IgM fractions were not protective.[28] Whether the presumptively protective IgG antibodies in the J5 antisera were directed to J5 LPS and cross-reactive with meningoccal LPS or were polyclonally induced and specific for the meningococcal LPS also remains undetermined. The demonstration by Siber et al.[31] of increases in ELISA titers of IgG antibody to meningococcal LPS in J5 rabbit antisera that were no more pronounced than the polyclonal responses seen to other LPS despite longer immunization schedules than those used by Davis et al.[28] favors the latter possibility. In any case, the observations are consistent with the concept that antibodies to core determinants can confer protection against LPS, providing that the core epitopes are unencumbered by O polysaccharide side chains.

Thus, several examples of binding to and protection against wild-type Gram-negative bacteria and their LPS by J5 antisera can be cited. However, these represent selective, not broad-spectrum, binding and protection. It is also possible that the protective serum factors were natural or polyclonally-induced antibodies directed not at common core epitopes but instead specific for each bacterium and LPS.

Re-evaluation of the evidence for broad-spectrum protection of animals by anti-lipid A antibodies in J5 and R595 antisera

Antisera generated by immunization with heat-killed J5 or R595 rough mutants possess only minimal increments in anti-lipid A titers.[31,32] Nevertheless, the possibility that such antibodies might account for the reputed broad protective activity of J5 and R595 antisera must be considered. In 1985, Dunn et al.[88] described a murine IgG1 MAb and Teng et al.[159] described a human IgM MAb, both of which reacted best with isolated J5 or R595 lipid A, and both of which broadly cross-reacted with wild-type Gram-negative bacteria and their LPS. The broad binding to bacteria is not surprising, since boiled organisms were used by Dunn et al. No data regarding this critical point were provided by Teng et al.

The reputed broad-spectrum binding of these two lipid A-reactive MAbs to all S-form LPS tested, however, is puzzling.[87,88,159] This implies that the lipid A epitopes in the LPS from these smooth strains are not masked by the O and core polysaccharides. Most other studies suggest that this is not the case. Lipid A-reactive MAbs prepared with the use of cells obtained from humans who possessed high titers of antibodies to J5 or R595 LPS were shown to be unreactive with a panel of LPS from smooth strains unless the lipid A was unmasked by acid hydrolysis.[85] Other studies with lipid A-reactive murine MAbs prepared with the use of J5 and R595 mutants have failed to demonstrate any broad-spectrum reactivity with S-form LPS, in contrast to their reactivity with whole boiled organisms.[84,86] The inability of polyvalent antibodies to lipid A, prepared by vaccinating rabbits with rough *Shigella sonnei* coated with its extracted lipid A, to bind to the parental S-form LPS was demonstrated by Lugowski and Romanowska.[160] Similarly, the inability of polyvalent antibodies to lipid A, prepared by vaccinating rabbits with the R595 mutant coated with its extracted lipid A, to bind to S-form LPS was demonstrated by Johns et al.,[32] by Mattsby-Baltzer and Kaijser,[161] and by Siber et al.[31] Freudenberg and Galanos have confirmed the masking of lipid A epitopes in S-form LPS and the exposure of these epitopes only after acid hydrolysis.[104]

The inaccessibility of lipid A epitopes on S-form LPS has also been shown with the use of hemolysis-inhibition assays[161] and immunofluorescent-antibody binding or RIA.[162,163] Moreover, the original contention by Galanos et al. that polyclonal rabbit antiserum to lipid A cross-reacted with all S- and R-form LPS tested in a passive hemolysis assay[164] appears to have been retracted, since the same laboratory subsequently reported that 'antibodies to lipid A show no interaction with intact LPS'.[165]

In addition to the masking of lipid A epitopes by O-specific carbohydrates, there is significant masking by core polysaccharides. Brade and Galanos reported that immunoadsorbent-purified rabbit antibodies to *S. minnesota* lipid A, prepared from serum of rabbits vaccinated with the R595 *S. minnesota* mutant coated with its extracted lipid A, failed to react with LPS from Rb, Rd, or Re chemotypes of *S. minnesota*, as assessed by passive immune hemolysis. Moreover, the *Salmonella* lipid A-anti-*Salmonella* lipid A passive hemolysis system could not be inhibited by LPS from Rb, Rd, or Re chemotypes of *Salmonella*. Only after these rough-mutant LPS underwent acid hydrolysis did epitopes capable of binding with lipid A antibodies become exposed.[166] Similarly, a murine MAb with high lipid A reactivity, prepared with the use of purified lipid A, was shown by Elkins and Metcalf to be virtually unreactive not only with smooth *S. typhimurium* LPS but also with LPS from *S. minnesota* mutants of Ra, Rb, Rc, Rd, or Re chemotypes.[163] Masking of lipid A-reactive binding sites by core carbohydrates of LPS has also been demonstrated by Kirkland et al. Murine MAbs, prepared with the use of J5 or R595 mutants, that were highly reactive with lipid A were shown to be virtually unreactive with LPS from an Ra chemotype of *S. minnesota* and to become progressively more reactive with LPS from increasingly rough chemotypes (Rc and Re *S. minnesota* mutants).[167] Comparable results were reported by Mullan et al., who used polyvalent rabbit antisera to lipid A and a series of increasingly rough *S. minnesota*

mutants.[162] Appelmelk et al. reported that two lipid A-reactive murine MAbs, prepared by immunization with the J5 mutant, failed to bind to LPS from Ra, Rb2, Rc, Rd1, and Re *S. minnesota* mutants or to J5 LPS.[72]

Pollack et al. prepared 17 J5 murine MAbs reactive with lipid A from J5 or R595 and assessed their binding in ELISA to a panel of LPS from 16 smooth strains that included 6 *E. coli* serotypes. One MAb bound weakly to the LPS from *K. pneumoniae* and *Pr. mirabilis* and another MAb also bound weakly to *Pr. mirabilis* LPS, but both failed to bind to any other S-form LPS. The remaining lipid A-reactive MAbs were incapable of binding to any S-form LPS. Moreover, with one exception, none of the 17 lipid A-reactive MAbs was capable of binding to LPS from Ra or Rb chemotypes of *S. minnesota* or from an Ra chemotype of *E. coli*. Additional studies with two of the lipid A-reactive antibodies in which passive hemolysis was employed yielded similar results.[73] More recently, Warren et al. reported no appreciable binding of two lipid A-reactive MAbs to S-form LPS of 10 *E. coli* serotypes.[168]

Thus, there is considerable support for the concept that lipid A-reactive antibodies do not bind to wild-type S-form LPS and that such lack of binding occurs primarily because the lipid A is masked by core and O polysaccharides. As an alternative explanation, it has been proposed that free lipid A, i.e. lipid A prepared by acid hydrolysis, represents a neoantigen expressing determinants not present in LPS.[169]

The differences between the observations by the numerous investigators cited above[31,32,72,73,84–86,104,160–163,165–168] and those of Dunn et al.[87,88] and Teng et al.[159] with respect to broad-spectrum binding of their lipid A-reactive antibodies to S-form LPS do not appear to be related to some unique ability of the antibodies prepared by the latter investigators to bind to the lipid A epitope. The MAb A6(H4C5) used by Teng et al. bound strongly to lipid A prepared from J5 LPS by acid hydrolysis, the standard method of exposing lipid A, and such lipid A also competitively inhibited binding of A6(H4C5) to Gram-negative bacteria.[159] Since virtually every study has failed to show broad-spectrum binding of lipid A-reactive antibodies to S-form LPS, we believe that additional data are required before the broad-spectrum binding of the MAb A6(H4C5) to S-form LPS (demonstrated by an immunoblotting technique) can be accepted as physiologically relevant. The need for such caution is underscored by a re-assessment by Bogard et al.[86] of the lipid A-reactive MAb reputed to exhibit broad-spectrum binding (8A1) employed by Dunn et al.[87,88] Whereas Dunn et al. reported broad cross-reactivity of 8A1 with S-form LPS in an ELISA, this MAb was unreactive with S-form LPS when assessed by a solid-phase RIA.[86]

As with the broad-spectrum reactivity in vitro attributed to the lipid A-reactive MAbs A6(H4C5) and 8A1 for enterobacterial S-form LPS, the broad-spectrum protective activity in vivo reputedly conferred by these MAbs in mice and rabbits[87,88,159] is also inconsistent with observations by most other laboratories. Other investigators have failed to demonstrate broad-spectrum protection against viable enterobacteria in animals actively immunized with lipid A.[16,17,44,162] Rabbit antiserum to lipid A also failed to transfer protection against isolated S-form LPS to rabbits unless the animals had been pretreated with LPS or lipid A.[170] Although the mechanism for (and significance of) this requirement for such pretreatment is unknown, the reputed broad-spectrum protection against lethality from S-form LPS by the lipid A-reactive MAb 8A1 cannot be based on this mechanism since the test animals were not pretreated with LPS or lipid A.[87,88] The other lipid A-reactive MAb, A6(H4C5), was considered protective because it prevented the dermal Shwartzman reaction in rabbits.[159] This capability, however, is not unique and indeed would be expected for lipid A-reactive antibodies since it involves pretreatment of rabbits with LPS (preparatory dose) and has been demonstrated with polyvalent rabbit antisera to purified lipid A.[170] While lipid A-reactive antibodies may protect rabbits against the dermal Shwartzman reaction, these antibodies appear incapable of directly neutralizing LPS,[72,170] or of protecting against lethality from challenge with viable Gram-negative bacteria.[16,17,44,162] Salles et al. confirmed the inability of a lipid A-reactive MAb to reduce murine mortality from challenge with LPS of smooth *E. coli*.[171]

On the basis of the above considerations, we believe that the broad-spectrum protective activity against lethality, from wild-type Gram-negative bacteria and their S-form LPS attributed to two lipid A-reactive MAbs, 8A1 and A6(H4C5),[87,88,159] could be mediated by mechanisms other than broad-spectrum binding to, and subsequent neutralization of, S-form LPS. First, and most importantly, no precautions were specified for ensuring that the MAbs 8A1 and A6(H4C5) or their controls were free of contaminating endotoxin.[87,88,159] Ensuring that these MAbs were endotoxin-free would be essential in those studies in which these antibodies were given 2–18 h before challenge,[159] since nonspecific enhancement of host resistance to infection and to LPS occurs rapidly after exposure to trace amounts of LPS.[172,173] Ensuring that the control preparations are free of endotoxin contamination is essential when bacterial or LPS challenge is performed immediately after administration of the test preparations,[87,88] since contamination of the control preparation with LPS can elevate mortality in the control group, making it appear that the J5 or R595 MAbs are protective.

Two studies emphasize the crucial importance of excluding trace endotoxin contamination before it is concluded that any MAb protective activity occurred. Chong

and Huston showed that MAbs to LPS core often contained trace amounts of contaminating LPS and that these preparations evoked protection regardless of the specificity of the MAbs. They observed that as little as 1 ng of LPS from *E. coli* O111:B4 given 2–96 h before challenge with viable *E. coli* protected mice against mortality, that even 0.1 ng could be protective, and that MAbs to the core region of LPS were not protective unless they were contaminated with such trace amounts of LPS.[174] Similar results were described by Woods et al. using MAbs against meningococcal challenge.[175]

In addition to the problem of endotoxin contamination, selection of the control MAbs warrants further consideration. The broad-spectrum protective effect of the MAb 8A1 was demonstrated with the use of 'sterile' saline as a control.[87,88] Not only is this an inadequate control, but 'sterile' saline can be highly contaminated with endotoxin. In the studies with MAb A6(H4C5) of protection against challenge with viable bacteria, the control consisted either of a human hybridoma containing the same amount of IgM as the MAb but with no anti-J5 activity or of 1.0 ml of Iscove's medium with 15% fetal calf serum; the control for the dermal Shwartzman reaction consisted of Iscove's medium.[159] We believe that not only should identically prepared MAbs to unrelated antigens have been employed for all the control studies involving the lipid A-reactive MAbs, but also a number of such control MAb preparations should have been tested before specific protective effects were assigned to any one J5 or R595 MAb. Thus, Miner et al. observed that a control MAb to hepatitis B surface antigen was capable of protecting mice against endotoxin lethality.[59]

The need for adequate controls is illustrated by recent studies by Fang et al.[176] A lipid A-reactive human IgM MAb was reported capable of broad-spectrum inhibition of the cytokine-releasing activities of S-form LPS incubated with human peripheral blood mononuclear cells in vitro, and of lethality when preincubated with S-form LPS before injection into galactosamine-sensitized rats. Except for one assay, in which the control consisted of a commercially prepared myeloma IgM, all assays were performed using buffered saline controls. The myeloma IgM control significantly inhibited LPS stimulation of cytokine production but, since the lipid A MAb inhibited to a greater extent, its neutralizing effect was regarded as 'specific'. Further studies will be needed to prove the specificity of this protection.

Finally, the caveat that some assays (e.g. ELISA) can be markedly affected by the nonspecific affinity of hydrophobic structures such as lipid A for MAbs[177] emphasizes the need for caution before acceptance of a conclusion that binding by J5 or R595 MAbs must reflect specific interactions with common core epitopes. Indeed, Pollack et al. assessed their findings that showed highly restricted cross-reactivities of J5 and R595 MAbs and concluded: 'the specificities of core and lipid A-reactive MAbs documented here are at variance with those described previously, which stress the broad cross-reactivity of such antibodies among heterologous and smooth LPS... This discrepancy may be explained in part by nonspecific binding'. The precautions necessary to minimize such nonspecific binding were carefully described in this study.[73]

Warren et al. recently re-assessed the ability of two lipid A-reactive MAbs to bind to and neutralize LPS in vitro. These MAbs, HA1A and E5, which have been used in clinical trials, exhibited no appreciable binding to or neutralization of S-form LPS. It was concluded: 'our experiments do not support the hypothesis that either of these anti-lipid A MAbs function by neutralizing the toxic effects of LPS'.[168] For the above reasons, it is highly improbable that the reputed broad-spectrum protection by J5 and R595 antisera against smooth Gram-negative bacteria and their LPS is mediated by lipid A antibodies.

One report may serve to illustrate some of the points discussed above. Dunn et al., having accepted the proposition that their lipid A-reactive murine MAb, 8A1, provided broad-spectrum protection to mice against Gram-negative bacterial sepsis on the basis of cross-reactivity with LPS, compared the efficacy of 8A1 with that of polyvalent J5 equine antisera. They reported that both 8A1 and J5 equine antisera were capable of broad-spectrum binding to smooth wild-type Gram-negative bacteria and their LPS and were equally protective in mice against challenge with *E. coli* O111:B4.[178] However, problems with methodology may account for these findings:

1. The broad-spectrum binding of the murine MAb 8A1 and polyclonal J5 equine antisera to smooth Gram-negative organisms was demonstrated with boiled organisms; binding to S-form LPS was demonstrated with the use of LPS that had been fractionated with SDS-PAGE.

2. Only a single challenge strain was employed: the wild-type parental strain (*E. coli* O111:B4). As already emphasized, immunization with the J5 mutant, as with R595, can evoke polyclonal rises in O-specific antibodies that are particularly marked against the parental smooth strain.[24,27,31–33,53,59,60] The J5 equine antisera employed by Dunn et al. appeared to possess such antibodies, as suggested by the illustrated results with SDS-PAGE. Specific O-antibodies were not quantitated, nor were their effects considered in assessing protection against the parental challenge strain.

3. None of the antibody preparations or the sterile-saline controls was examined for endotoxin

contamination, nor were precautions indicated for circumventing such contamination.

4. Protection by the J5 antibody preparations was judged solely by comparisons against sterile saline-treated controls; comparably prepared control MAbs were not utilized for assessment of the efficacy of 8A1, nor was matched pre-immune serum from the same equine donor used for assessment of the polyvalent J5 antiserum. Normal equine sera have been shown to protect mice against challenge with enterobacteria.[35]

These crucial points must be considered in conjunction with the inability of MAb 8A1 to bind in RIA to intact S-form LPS[86] and the fact that most previous studies have demonstrated that lipid A-reactive antibodies can neither bind in broad-spectrum fashion to S-form LPS or to non-boiled smooth organisms nor protect against enterobacterial infections. Thus, we conclude that the protection reported with MAb 8A1 and with polyclonal equine J5 antisera against challenge with the parental *E. coli* strain[178] has not been proven to be mediated by cross-reactive antibodies to R-form LPS.

Re-evaluation of the evidence for broad-spectrum protection of animals by human antisera to J5 and R595

Several studies have examined the protective efficacy of human antisera and/or their immunoglobulin fractions raised against J5 or R595 in animal models of Gram-negative bacterial sepsis.[29,62,117,153] Martinez and Callahan reported that human antiserum to J5 was unable to protect immunosuppressed (leukopenic) mice against challenge with *P. aeruginosa* serotype 5; rates of survival among untreated animals and among those treated with pre-immune and postimmune J5 sera were 5%, 63%, and 58%, respectively.[117] In contrast, McCabe's laboratory reported that human antisera to R595 protected mice against challenge with viable *K. pneumoniae* and *M. morganii* and against challenge with *S. typhi* LPS.[29,62] These findings were considered affirmation of broad-spectrum protection by antibodies to inner-core LPS epitopes.[29,62,121] However, pre-immune sera from a majority of normal humans were found capable of protecting mice against the two challenge strains of viable enterobacteria; postimmune R595 sera from these subjects were found to be unsuitable for testing for increments in protective activity because, as stated by the investigators: 'demonstrating any enhancement of protective activity after Re immunization in subjects whose preimmunization serum demonstrated protective activity...was impossible'.[62] Only postimmune sera of subjects whose pre-immune sera lacked protective activity were shown to develop increments in protective activity after R595 vaccination. The latter findings were taken as evidence for the broad-spectrum efficacy of antibodies to R595 LPS. The former findings were dismissed with the speculation that type-specific natural antibodies accounted for the protective effects of pre-immune sera and that these precluded detection of any additional protection by the R595 LPS antibodies raised by vaccination.[29]

Two alternative hypotheses, however, must be considered: (i) that the protection conveyed by the majority of normal pre-immune human sera might be based on factors other than type-specific antibodies, that these unidentified protective factors increased following R595 vaccination, and that increments in protection were more readily observed when these factors were initially absent; and (ii) that type-specific antibodies did indeed account for protection by the majority of pre-immune human sera and that in subjects whose pre-immune sera lacked such antibodies (and hence provided no protection), these antibodies were polyclonally stimulated by the R595 vaccination but were not serologically detected by the assay employed. As analyzed below, the data presented are consistent with one or both of these alternative mechanisms:

1. No correlation was found between titers of antibody to R595 LPS and protective activity of R595 antisera. In fact, those human antisera to R595 with the lowest titers of HA antibody to R595 LPS (< 1:10) provided mice the greatest protection against challenge with viable *K. pneumoniae* or *M. morganii*.[62] In addition, when the R595 vaccine was stored for long periods before use, the resulting human antisera were no longer protective even though the ability of the vaccine to stimulate production of antibody to R595 LPS remained intact.[62] Furthermore, following immunization, no close temporal correlation existed between titers of circulating antibody to R595 LPS and protective activity. Finally, attempts to show correlations between protective activity and antibodies to R595 LPS by separate analysis of IgG and IgM titers were unsuccessful.[62]

2. The protection afforded mice by human R595 antisera against the Caroli strain of *K. pneumoniae*, as reported by McCabe et al., was discernible only if the pre-immune serum lacked protective activity. Yet we observed that rabbits whose pre-immune sera lacked significant protective activity in mice for the same strain of *K. pneumoniae* failed to develop any increments in serum protective activity following immunization with R595. This excluded the possibility that any protective activity of antibodies raised by immunization with R595 may have been masked by protective factors demonstrable in most normal rabbit sera.[35]

3. Sephadex G-200 was used to prepare the protective IgM fractions from R595 antisera. These fractions were administered to animals intravenously 1 h before challenge with the viable *K. pneumoniae* or *M. morganii* or with the *S. typhi* LPS. No precautions were specified for circumventing contamination of these fractions with LPS, and no data were presented to prove its absence.[29] We have observed that IgM serum fractions prepared with Sephadex G-200 are consistently contaminated with variable quantities of LPS and that the broad-spectrum protective activity attributed by earlier investigators to IgM antibodies against common LPS toxophore groups could be accounted for by such contamination.[172] We have also specified the precautions necessary to ensure that protective activity of Sephadex G-200 serum fractions is not attributable to the artifact of LPS contamination.[53] McCabe et al. have argued that LPS contamination could not account for the broad-spectrum protection conferred in their studies because R595 antiserum was given 1 h before challenge with the viable organisms or S-form LPS, whereas 'LPS induces increased susceptibility in the first 18 hours, after which increased resistance to a number of types of lethal challenge occurs'.[29]

   Landy and Pillemer, however, observed that the time of onset of increased resistance of mice to bacterial challenge after LPS administration can be highly variable and that one crucial determinant is LPS dose; if small quantities of LPS (10, 1, or 0.1 μg) are given, delay in development of resistance can be reduced to a few hours, and during this induction phase there may be no evidence of increased susceptibility.[179] Chong and Huston confirmed these findings using nanogram quantities of LPS.[174] More pertinent are the earlier data of Parant et al.,[152] who found that minute amounts of endotoxin (0.01 μg) markedly protected mice when given only 10 min before challenge with the Caroli strain of *K. pneumoniae* used by McCabe's group. These findings, together with the inability of our laboratory[35] and of Ng et al.[37] to demonstrate protection by rabbit antisera to R595 of mice challenged with the Caroli strain, raises the possibility that the protection against this viable strain reported by McCabe et al. afforded by their Sephadex G-200 IgM fractions of rabbit and human antisera to R595[29] might be attributable to contamination with endotoxin.

   Even the protection observed against *S. typhi* LPS by the R595 IgM serum fractions may be related to endotoxin contamination, since the LPS was combined with actinomycin D to sensitize mice to the lethal effects of LPS.[29] Mice challenged intravenously with LPS in combination with another sensitizing agent, D-galactosamine, are markedly protected if they are given trace amounts of endotoxin (0.1 μg) intravenously 1 h before challenge.[180] Studies of the effects of endotoxin pretreatment of actinomycin D-sensitized mice and quantification of endotoxin in the IgM fractions will be required for resolution of these issues.

4. No studies were performed that proved the specificity of the protective activity generated by R595 immunization. For example, no studies used whole sera or Sephadex G-200 fractions of whole sera obtained from volunteers vaccinated with wild-type smooth Gram-negative organisms to evoke comparable local and systemic inflammatory and febrile responses without producing increments in antibodies to R595 epitopes.

5. The possibility that polyclonal serotype-specific antibodies, rather than antibodies to R595 LPS, in R595 antisera could account for protection was excluded on the basis that titers of specific antibodies 'did not increase materially' in human sera as assessed by HA assays.[29,62] This is not definitive evidence. We have observed that increments in O antibodies that are undetectable by agglutination assays could be detected by more sensitive techniques, i.e. modified Farr and bactericidal assays, and can confer O-specific resistance to LPS.[181] It is relevant in this regard to emphasize that no data were presented to show that the natural type-specific antibodies postulated as mediators of the broad-spectrum protection by pre-immune sera from the majority of human donors could consistently be detected by the HA assay employed.[29] Given the marked sensitivity of one of the challenge strains to serotype-specific antibody (mice were well protected against lethality from challenge with the Caroli strain by 10^{-4} ml of specific antiserum[11]), the appearance of protective activity in R595 antisera despite the apparent lack of 'material' increases in levels of serotype-specific antibodies could be based simply on insensitivity of the HA assay employed.

We conclude that the two reports on human antisera to R595 by McCabe's group[29,62] do not provide definitive evidence for the hypothesis that the ability of such antisera to protect mice from the Caroli strain of *K. pneumoniae*, from *M. morganii*, or from *S. typhi* LPS is based on the activity of cross-reactive antibodies to R595 LPS.

Nys et al. reported that IgG and IgM fractions prepared by ammonium sulfate precipitation from human blood donors displaying high ELISA titers against J5 or R595 LPS (> 1:6400) protected actinomycin D-sensitized mice

against lethality from the LPS of *E. coli* O7:K1:H6, whereas fractions with low titers (< 1:400) did not do so.[153] The protective serum fractions, however, failed to provide significant protection against the S-form LPS of *S. enteriditis*. In an earlier section, we have discussed the evidence that *E. coli* O7:K1 possesses rough or semi-rough LPS, rather than the S-form type. The observations by Nys et al., just as those of Flynn et al.,[112] can be accounted for by binding of anti-core antibodies to presumptive rough or semi-rough LPS in *E. coli* O7:K1.

Re-evaluation of the evidence for broad-spectrum protection in humans by human antisera to J5 and R595

Two clinical trials indicated that human antiserum to J5 conveys significant broad-spectrum protection against mortality due to Gram-negative bacterial sepsis.[25,26] The data presented in this review, however, raise serious questions regarding the hypothesis that such protection is based on broad-spectrum neutralization of LPS by antibodies to J5 core. Two important alternatives to this hypothesis discussed in previous sections, i.e. protection by natural or by polyclonally stimulated serotype-specific antibodies and protection by acute-phase serum factors, have not been excluded.

Not only do different normal human sera contain various and significant titers of natural serotype-specific antibodies to a variety of S-form LPS, but such titers may greatly exceed those raised to core LPS in the J5 antisera employed in clinical trials.[182–185] Indeed, because of the prevalence of such natural antibodies, Gaffin advocated the use of mixtures of serotype-specific antibodies to LPS from normal human plasma for treatment of Gram-negative bacterial sepsis.[185] Consequently, even though pre- and postimmune J5 sera from the same volunteers were given in one 'positive' clinical trial,[25] valid conclusions would have been possible only if the protective effects of natural serotype-specific antibodies had been adequately controlled by testing each set of paired sera during infections caused by identical bacterial serotypes. Furthermore, it would have also been necessary to carefully exclude the protective effects of increments in polyclonal serotype-specific antibodies induced by the J5 immunization. The importance of limiting comparisons of protection by any given set of pre- and postimmune sera to identical bacterial serotypes is further emphasized by the finding that antibodies to *Streptococcus pneumoniae*, which may have been present in some of the human sera, can cross-react with some enterobacterial surface antigens (*P. aeruginosa* LPS and K antigens of *E. coli* and *Klebsiella*) and offer cross-protection against enterobacterial sepsis.[115,186] In addition, variable levels of antibodies to Pseudomonas exotoxin A are present in normal human sera, and these can protect against *P. aeruginosa* septicemia.[184,187–189]

We recognize that assessment of protection by matched pre-immune and postimmune antisera to rough mutants against identical Gram-negative bacterial serotypes is not feasible in most clinical trials. However, it would be feasible to provide assurances that the distribution and titers of serotype-specific antibodies to the infecting bacterial strains in the pre- and postimmune J5 sera were comparable before attributing protection to antibodies to core LPS epitopes. Assurances of comparable distribution and titers of other antibodies with proven protective activity against the infecting bacterial strains, such as antibodies to Pseudomonas exotoxin A [187,188] and to capsular antigens,[39,42,44,115,190–192] would also be important, particularly in view of the frequency of infections with Pseudomonas and encapsulated enterobacterial strains. Indeed, on the basis of clinical studies that correlated survival with titers of serotype-specific IgG antibodies[184,193] or of antibodies to Pseudomonas exotoxin A [184,189] at the onset of Gram-negative bacterial sepsis, such assurances would appear to be as important as those regarding the comparable distribution of the nature and severity of underlying clinical illness. Such assurances, however, were not provided in the clinical trials conducted by Ziegler et al.[25] and by Baumgartner et al.[26]

It could be argued that since serious sepsis due to Gram-negative bacteria in humans can be produced by rough-mutant organisms and since the cumulative experimental evidence presented in this review militating against significant broad-spectrum protection by antisera to inner core LPS epitopes almost always involved studies with smooth organisms, extrapolations to human sepsis may not be valid. If autoagglutinability of strains isolated from blood cultures is selected as an indicator, rough strains may cause 4–15% of the cases of *E. coli* bacteremia in humans.[194,195] In other reports, rough strains comprised 4% of 149,[196] 5.5% of 288,[197] and 3% of 239 *E. coli* bacteremic isolates.[197] A later study employed SDS-PAGE and rough-specific phages to identify Gram-negative bacteria with R-form LPS phenotypes; all but one of the Gram-negative strains isolated from the blood of 17 patients were found to be smooth.[63] These data are in agreement with the finding that rough organisms are almost invariably serum-sensitive[100] and that 'isolates from systemic human clinical infections are almost uniformly resistant,> 85%, to killing by serum'.[99] Since available evidence suggests that rough organisms with the more-deficient core LPS are even less virulent than less-deficient rough strains,[67] it would be anticipated that those with the more complete core (Ra and Rb chemotypes) might predominate within the rough-septic group. Evidence that antibodies to the inner core LPS epitopes in such superficial rough mutants can be blocked from binding by the outer

core polysaccharides has been cited earlier in this review. Therefore, if antibodies to core LPS epitopes are protective against human infections with rough chemotypes, it might be difficult to discern the impact on overall mortality of antibodies reactive with the deeper inner core epitopes raised by vaccination with rough mutants such as J5 and R595.

Cross et al. have emphasized that among *E. coli* isolates, strains with R-form LPS represent a larger proportion of rough phenotypes than can be detected by autoagglutinability; this is caused by the masking action of capsular polysaccharides.[113] We are unaware of critical studies on the effectiveness of J5 or R595 antisera against encapsulated, phenotypically rough *E. coli* strains, particularly in conjunction with optimal doses of capsular-disruptive antibiotics. However, even if effective, the overall impact on mortality might still be difficult to detect, since the proportion of such rough strains among patients with bacteremia ranged between 6 and 11%.[43,113] (The latter figure is estimated from reports that *E. coli* comprised 40% of all cases of nosocomial bacteremia, and that 28% of bacteremic *E. coli* were sensitive to rough-specific phages.[113])

An additional and important consideration in clinical trials with antiserum to rough mutants is the potential contribution of acute phase serum factors to broad-spectrum protection. As reviewed in an earlier section, acute-phase serum factors which are elicited by IL-1 possess LPS-neutralizing activity. Depending on the immunization schedule and time of bleeding, immunization of volunteers with J5 or R595 vaccines might induce production of acute-phase serum factors of therapeutic significance.[198]

It is apparent that multiple factors may contribute to protective activity of antisera raised by immunization with rough mutants. These include acute-phase serum proteins and natural and polyclonally-induced serotype-specific antibodies to LPS of smooth Gram-negative bacteria, their capsules, and their exotoxins. Although antibodies to LPS core epitopes in antisera to J5 and R595 do not appear capable of providing broad-spectrum protection, they could contribute by providing a limited spectrum of protection as a consequence of infection with rough-mutant strains or of selective reactivity with LPS of smooth Gram-negative bacteria. It would be expected that in different clinical trials, involving different immunization schedules and distributions of Gram-negative bacterial species and strains, the relative contribution of each of these factors to protection by antiserum to rough mutants would be variable. These considerations could explain the inability to detect any significant relation between the protection conferred by antisera to J5 and titer of antibody to J5 core glycolipid in one 'positive' clinical trial.[25] This relation was not assessed in the second 'positive' J5 clinical trial; in fact, no antibody data were provided.[26]

The concept that antisera to Rc (J5) and Re (R595) rough mutants do not provide significant broad-spectrum protection is supported by the failure of five more recent clinical trials to demonstrate protection by J5 or R595 antisera or their immune globulin fractions. All these trials were randomized and, except for the first, double-blinded. In the first of these negative trials, pre- and postimmune J5 antisera were given prophylactically to patients with neutropenia; there was no difference in rates of Gram-negative bacteremia, febrile episodes, or mortality.[199] In a second negative clinical trial, a gamma globulin fraction prepared from donors with elevated antibody titers to J5 LPS proved no more effective than fractions with lower titers in preventing Gram-negative bacterial infections when given immediately before induction of aplasia in patients with leukemia.[200] In a third trial, an IgG fraction (200 mg/kg) prepared from the sera of volunteers immunized with the *E. coli* J5 mutant provided no more protection against mortality when given to patients with Gram-negative septic shock than did the IgG fraction prepared from a standard plasma pool. Furthermore, the J5 immunoglobulin did not reduce the number of systemic complications of shock and did not delay the occurrence of death from systemic shock.[201] In a fourth trial, infusions of human immunoglobulin preparations selected for their high content of IgG to R595 LPS afforded no greater protection against subsequent Gram-negative bacterial infections or their systemic complications in patients at high risk after major surgical procedures than did comparable immunoglobulin preparations containing on average 7-fold lower amounts of anti-R595 IgG.[202] In a fifth trial, 73 children with severe infectious purpura, the majority secondary to *N. meningitidis*, received J5 immune or preimmune plasma. It was concluded that the anti-J5 plasma did not affect the clinical course, the rate of decrease of TNFα and IL-6, or the mortality.[203]

CONCLUSIONS

The hypothesis that antibodies to common core epitopes in antisera raised against rough-mutant enterobacteria mediate broad-spectrum protection against wild-type enterobacteria and their S-form LPS currently rests primarily on observations that J5 and R595 antisera and their immunoglobulin fractions can confer such protection and that adsorption of these antibodies removes protective activity. We have reviewed the evidence that the controls for these observations were often inadequate and that broad-spectrum protection could not be obtained by our laboratory or by many other investigators. Alternative protective mechanisms have been considered; these include activity of natural and polyclonally-stimulated antibodies to O-specific and capsular antigens and to Pseudomonas

exotoxin A, presence of acute phase serum proteins, and contamination with endotoxin. Although antibodies to core LPS epitopes in antisera raised to Rc (J5) or Re (R595) chemotypes do not appear capable of providing broad-spectrum protection, they may provide a limited spectrum of protection as a consequence of selective cross-reactivity with S-form LPS or of infection with rough-mutant strains. This review has focused upon the inability of polyclonal antibodies to the inner core of LPS in rough-mutant antisera to confer broad-spectrum protection against endotoxemia and Gram-negative bacterial sepsis caused by smooth wild-type strains. It may provide a basis for future reviews of broad-spectrum protection by MAbs to LPS determinants.

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